New approaches to overcome cancer cell resistance and survival

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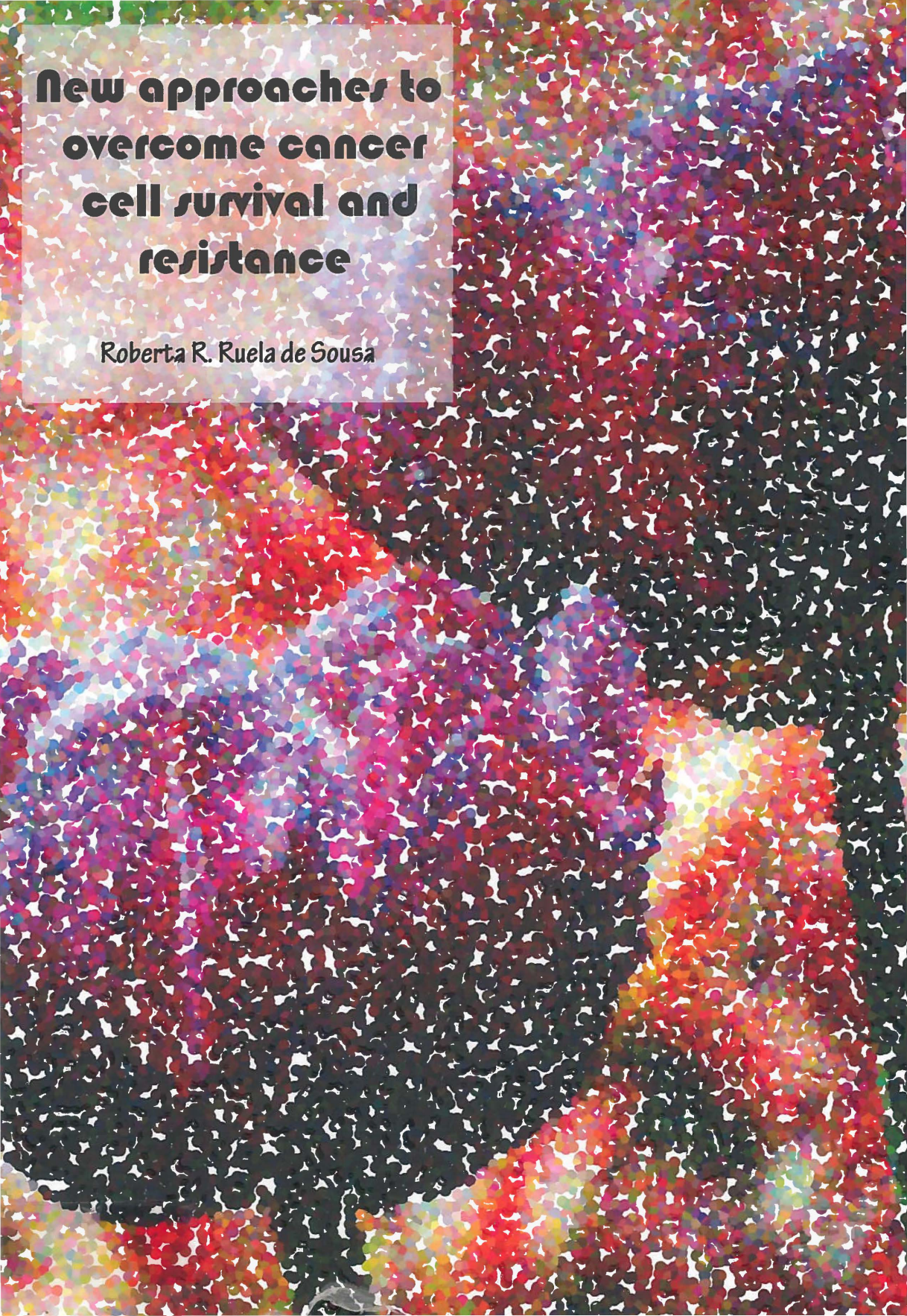
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Thesis University of Groningen with summary in Dutch

The studies described in this thesis were financially supported by the Groningen University Institute for Drug Exploration (GUIDE)

Printing of this thesis was financially supported by: Rijksuniversiteit Groningen, University Medical Center Groningen, Groningen University Institute for Drug Exploration (GUIDE),

Cover Photo by R.R. Ruela de Sousa
Printed by Wöhrmann Print Service

ISBN: 978-90-367-4860-5

Stelling behorende bij het proefschrift

New approaches to overcome cancer cell resistance and survival

1. Nature remains an endless source of medical drugs. (this thesis)
2. Protein Phosphorylation controls a wide number of cellular events and arose as potential target of therapy. (this thesis)
3. Although cancer cells were shown to display an excessive phosphorylation of certain signaling transduction proteins, the importance of phosphatases has so far been neglected. (this thesis)
4. Hedgehog signaling might be interesting for overcoming MDR resistance in myeloid leukemia. (this thesis)
5. In Biology, it is delightful to understand a biological process, but it is equally interesting to understand how and why it runs out of control.
6. "Only through curiosity can we discover opportunities, and only by gambling can we take advantage of them". *Clarence Birdseye*
7. "Science has promised us truth. It has never promised us either peace or happiness". *Gustave le Bon*
8. "There are no shortcuts to any place worth going". *Beverly Sills*

Roberta R. Ruela de Sousa, 20 June 2011

CMB

RIJKSUNIVERSITEIT GRONINGEN

New approaches to overcome cancer cell resistance and survival

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 20 juni 2011
om 11.00 uur

door

Roberta Regina Ruela de Sousa
geboren op 1 juni 1982
te Campinas/ SP, Brazilië

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Kaushal B. Parikh

Venham enfim as altas alegrias,
As ardentes auroras, as noites calmas,
Venha a paz desejada, as harmonias,
E o resgate do fruto, e a flor das almas.
Que venham, meu amor, porque estes dias
São de morte cansada,
De raiva e agonias
E nada.

José Saramago

CONTENT

Chapter 1

Introduction

INTRODUCTION

Cancer is the most public health problem in developed countries and is a rapidly increasing problem in developing countries [1]. GLOBOCAN, based on the International Agency of Research on Cancer (IARC), estimated 12.7 million new cases of cancer in 2008 and 7.6 million deaths. The estimative in the United States is 1,5 million new cases and more than 500 thousand deaths from cancer in 2010 [2]. This complex disease affects different tissues, displays uncontrolled cell growth, cell invasion to adjacent tissue and sometimes, metastasis. The very coordinated sequence of cellular events that keeps homeostasis in the body is broken in cancer cells and a new symphony regulates the proliferation and survival of cancer cells. In the last few years, considerable progress has been achieved concerning cancer biology. However, achieving an efficient treatment with low side effects, which targets specifically cancer cells and overcomes resistance, remains a challenge.

Searching for molecular targets in cancer cells has been an excellent strategy. Protein phosphorylation arose as a potential target of therapy, once cancer cells were shown to display an excessive phosphorylation of certain signaling transduction proteins. Reversible phosphorylation, a post-translational modification which controls every event in the cell physiology, is mediated by protein kinases and phosphatases. The protein kinases catalyze the transference of phosphate groups from ATP to hydroxyl groups of serine, threonine, or tyrosine amino acid residues. In contrast, the protein phosphatases catalyze the hydrolysis of phosphorylated amino acid residues. Some protein kinases are highly active in different cancer types, mainly due to fusion genes and gain of function mutations. For instance, 30% of acute myeloid leukemia (AML) display an activating mutation of the FLT3 receptor tyrosine kinase which is associated with a poor prognosis [3]. BCR-ABL fusion protein, an active kinase, is present in almost all patients with Chronic Myeloid Leukemia (CML) and enhances proliferative capability and resistance to apoptosis of hematopoietic stem or progenitor cells [4]. In fact, the first clinically successful tyrosine kinase inhibitor was imatinib mesylate (gleevec, approved in May 2001), a pioneer of a new approach to treat cancer patients [5]. Imatinib mainly inhibits ABL, and kills myeloid cells containing the BCR-ABL mutation without affecting on normal cells. Imatinib therapy is generally well tolerated, and minimal side effects are observed compared with other chemotherapies [4]. Targeting BCR-ABL with specific kinase inhibitors such as imatinib has drastically improved survival of CML patients [6].

In addition, imatinib also inhibits the c-Kit and PDGFR, and may therefore find a role in the treatment of gastrointestinal stromal tumor (GIST) formation, in which these tyrosine kinases are deregulated [7].

As kinases are upregulated in many types of cancer, it is logical that they are considered as targets of therapy. The first approach to counterbalance kinase activity is by increasing protein phosphatase activity. As such, phosphatases can be valuable targets for therapy and may help to understand the failure of kinase inhibitors therapies [8]. A notable example of phosphatase as target of therapy is the phosphatase and tensin homolog PTEN that is frequently mutated or deleted in a wide variety of solid tumors [9]. *PTEN* mutation has been associated with 5–27% of localized and 30–60% of metastatic prostate tumors [10]. This dual specificity phosphatase, acting as both lipid and protein phosphatase, classically converts phosphatidylinositol 3,4,5- phosphate (PtdIns(3,4,5)P3) at the plasma membrane to phosphatidylinositol 4,5- phosphate (PtdIns(4,5)P2). Thereby, PTEN directly antagonizes the activity of phosphatidylinositol 3-OH kinase (PI3K) and inhibits AKT activation, a serine-threonine kinase which is involved in proliferation, migration, survival and antiapoptotic pathways [11]. Hence, delivery of PTEN via gene therapy has shown promise, in combination with other type of therapies [11].

Despite the identification of specific kinase and phosphatase-related treatment options, many other types of cancer remain without a specific treatment. Moreover, most kinase inhibitors are not as specific as imatinib, thereby causing adverse effects. The pursuit of improved cytotoxic agents remains a considerable focus in the discovery of new anti cancer drugs.

Nature is not only a great source of pharmaceutical agents, but also of chemopreventive compounds. Fruits, vegetables and teas have been shown to possess cancer preventing properties, at least in part due to their high content in polyphenols [12]. Flavonoids and phenolic acids represent the majority of polyphenols present in food [13]. In the last few years, numerous studies have shown the cancer-preventing properties of flavonoids, using both cancer cell lines and animal tumors as model systems [14-16]. For instance, the flavonoid fisetin (3,7,3',4'-tetrahydroxyflavone) exerts antiproliferative effect on the human prostate cancer cell lines PC3 [17] and LNCap [17]. Fisetin also inhibits tumor growth and reduces serum prostate specific antigen (PSA) levels in athymyic nude mice implanted with Androgen Receptor-positive human prostate cancer cells [15]. Another flavonoid, apigenin, 4′, 5, 7,-trihydroxyflavone, has been effective against a wide variety of cancer cells such as breast [18], colon [19], ovarian [20] and prostate [21] cancer.

Another class of natural compound widely investigated for their biological activities is the terpenoids. In particular the diterpenoid ferruginol has been described as a promising anticancer agents due to its effect on leukemia [22] and prostate cancer cell lines [23, 24]. Additionally, ferruginol displays different biological properties such as anti-ulcerogenic [25], cardioactive [26], and anti-oxidative [27] properties.

Throughout human history, natural products have been used for many different applications in medicine. With regard to cancer, a wide number of important new clinical drugs have been obtained from natural sources. A new drug can be obtained by purification of active compounds from a specific plant, by structural modification of natural compounds or by synthesis of new compounds designed based on a natural compound [28]. For example, Paclitaxel (Taxol®) is a diterpene found in leaves of several species of *Taxus* and used in treatment of drug-refractory metastatic ovarian cancer. Paclitaxel was first isolated from the bark of the yew *Taxus brevifolia*, a tree native to western North America. As the amount of paclitaxel in Taxus is very low, the semisynthesis from another natural compound 10-deacetyl-baccatin III was required as an alternative. [28]. Docetaxel (Taxotere®) is an improved version of paclitaxel with better pharmacological properties and potent anticancer activity against several human cells. These clinical compounds exemplify the role of natural compounds as a viable source of new drugs.

Despite the continuous discovery of anticancer agents, some patients do not respond to treatment due to acquisition of multidrug resistance (MDR) against chemotherapy. Several molecular and cellular mechanisms might be involved in MDR, such as alteration in DNA repair, defective regulation of apoptotic gene expression, enhanced intracellular drug detoxication and overexpression of membrane drug transport proteins (eg. P-glycoprotein, P-gp) [29]. Thus, the development of resistance is the second challenge, after find a chemotherapeutic drug, in the clinical management of cancer.

Outlines of this thesis

The general goal of this thesis is further our understanding into cancer biology regarding dysregulation of signaling pathways in pathogenesis and resistance, in addition to proposing alternatives for chemoprevention and development of new drug agents.

This thesis will initially discuss the role of phosphatases in the major signaling pathways involved in hematological malignancies, i.e. the Ras-Raf-ERK, Jak-Stat and PI3K-PKB-mTOR pathways (Chapter 2). This is following

by a discussion on the involvement of Hedgehog signaling in the maintenance of chemoresistance in myeloid leukemic cells (Chapter3). Next, we describe our studies into the effect of three natural compounds in cancer cell survival. The first natural compound studied was the flavonoid fisetin (Chapter 4) which causes death of myeloid leukemia HL60 cells through reduction of ERK phosphorylation and an increase of p38 and JNK phosphorylation. The flavonoid apigenin (Chapter 5) induced apoptosis in HL60 and autophagy in TF1 cells. The last natural compound described is ferruginol (Chapter 6), which promotes downregulation of Ras/PI3K and STAT 3 and 5, resulting in cell cycle arrest and apoptosis in prostate cancer cells.

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Chapter 2

Reversible phosphorylation in haematological malignancies: potential role for protein tyrosine phosphatases in treatment?

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Maikel P. Peppelenbosch, Gwenny M. Fuhler

Biochim Biophys Acta. 2010, 1806:287-303

ABSTRACT

Most aspects of leukocyte physiology are under the control of reversible tyrosine phosphorylation. It is clear that excessive phosphorylation of signal transduction elements is a pivotal element of many different pathologies, including haematological malignancies and accordingly, strategies that target such phosphorylation have clinically been proven highly successful for treatment of multiple types of leukemias and lymphomas. Cellular phosphorylation status is dependent on the resultant activity of kinases and phosphatases. The cell biology of the former is now well understood; for most cellular phosphoproteins we now know the kinases responsible for their phosphorylation and we understand the principles of their aberrant activity in disease. With respect to phosphatases, however, our knowledge is much patchier. Although the sequences of whole genomes allows us to identify phosphatases using *in silico* methodology, whereas transcription profiling allows us to understand how phosphatase expression is regulated during disease, most functional questions as to substrate specificity, dynamic regulation of phosphatase activity and potential for therapeutic intervention are still to a large degree open. Nevertheless, recent studies have allowed us to make meaningful statements on the role of tyrosine phosphatase activity in the three major signaling pathways that are commonly affected in leukemias, i.e. the Ras-Raf- ERK1/2, the Jak-STAT and the PI3K-PKB-mTOR pathway. Lessons learned from these pathways may well be applicable elsewhere in leukocyte biology as well.

1. INTRODUCTION

Haematological malignancies account for approximately 10% of cancer in adults and 40% of pediatric cancers [1]. Annually, more than 100,000 new cases of leukemia, lymphoma or myeloma are diagnosed in the US alone, resulting in 60,000 deaths per year [2]. A host of genetic differences and molecular aberrations affecting blood cells at different stages of differentiation lead to a wide range of haematological tumors with different clinical manifestations. Many haematopoietic signaling pathways are regulated by protein and lipid phosphorylation, a process generally mediated by kinase activity. As proper regulation of phosphorylation events is critical for cellular homeostasis, dysregulation of activity of a single protein kinase can result in oncogenic transformation, characterized by increased cell division, growth, decreased differentiation and/or cell death [3]. Indeed, numerous protein kinases have well-defined, causative roles in human haematological malignancies. Several of these fall into the class of receptor tyrosine kinases (RTK). For instance, activating mutations of the FLT3 RTK are found in 30% of Acute Myeloid Leukemia (AML) patients and contribute to myeloid pathogenesis [4]. Others include activating point mutations or the generation of activating fusion partners due to diverse translocations involving the platelet derived growth factor (PDGF) receptor, c-KIT or the fibroblast growth factor (FGF) receptor (reviewed in [5]). In addition, inappropriate activation or over-expression of non-receptor kinases have been reported in a wide range of haematological malignancies.

Interestingly, most kinase deregulations observed in haematological cancers seem to affect one or more of three major signaling pathways:
i) the Ras-Raf-MEK- extracellular signal regulated kinase (ERK1/2) pathway;
ii) the Jak-signal transducer and activator of transcription (STAT) pathways;
iii) the phosphatidylinositol kinase (PI3K)- protein kinase B (PKB/AKT)-mammalian target of Rapamycin (mTOR) pathway [6].

Aberrant expression or activity of kinases within these pathways themselves has been described independent of disturbed RTK signaling. The balance between activation and de-activation of signaling pathways is a delicate one, which is regulated not only through phosphorylation by kinases, but also through de-phosphorylation events induced by a diverse range of phosphatases. Intuitively, by counterbalancing the activity of kinases, phosphatases should primarily act as tumor suppressors. However, novel regulatory actions of phosphatases in cancer are emerging, not only as a

negative modulator of kinase activity but also as causative agents in themselves [7].

Although the role of aberrant kinase activity and the potential for tyrosine and serine/threonine kinase inhibitors in the treatment of various leukemias have been investigated and reviewed extensively [8-11], the role of phosphatases in haematological malignancies has received less attention to date. In this review, we address the mechanisms by which some phosphatases may affect haematological malignancies and discuss their potential in therapeutic treatment. We explore general characteristics of those phosphatases, their role in the signaling pathways in the homeostatic cell and the result of their dysregulation in the progression or suppression of haematological malignancies.

2. PROTEIN PHOSPHATASES

Two major families of protein phosphatases can be distinguished based on their substrate specificity; the serine/threonine phosphatases (STP) and protein tyrosine-phosphatases (PTP) (Table 1). Recently, a third class of protein histidine-phosphatases has emerged, but relatively little information is available on this group of phosphatases (reviewed in [12]). Of the 148 human protein phosphatase genes recognized, only 30 encode for STP [12-13]. Despite there being only few STPs, more than 400 genes coding for serine/threonine kinases are identified, accounting for the majority of protein phosphorylation. This suggests a large degree of substrate-promiscuity of STPs. For this reason, although alterations in PPP family members have been described in (haematopoietic) cancer cells, and STPs appear to be viable cancer therapy targets [14-15] this group will not be further discussed in this review.

Of the 107 PTPs, only 81 are thought to be catalytically active and dephosphorylate phosphotyrosine residues, corresponding to 90 protein tyrosine kinases, of which around 80 are suggested to be catalytically active. This implies that there is a comparable substrate specificity of PTKs and PTPs, and an equal contribution in regulating tyrosine phosphorylation patterns. This is further suggested by the parallel tissue distribution of PTPs and PTKs, with hematopoietic tissues expressing the highest proportion of all PTPs [16]. Within the PTPs, different classes are recognized based on amino acid sequence in the phosphatase catalytic domains. Class I is by far the largest and can be subdivided into classical PTPs and dual specificity PTPs (DUSP), which aside from dephosphorylating both tyrosine and serine/threonine residues also recognize other substrates. The classical PTPs can be further separated into receptor PTPs (RPTP), and non-receptor PTPs (NRPTP), whereas the DUSPs are separated into 7 distinct categories (Table 1). Of these, the MKPs show specific activity towards mitogen-activated protein (MAP) kinase-ERK1/2 pathway, and are as such of particular interest in tumors where over-activation of this pathway is found [17]. Of particular interest is phosphatase and tensin homolog (PTEN), which is ordered under the DUSPs as it contains the cysteincased motif that classifies PTPs, [18] but seems to have a higher specificity for the 3 position of the inositol ring of the lipid phosphatidylinositol 3,4,5 phosphate (PtdIns(3,4,5)P3), and can therefore also be classified as a lipid phosphatase [19-20]. Lipid phosphatases generally counteract the role of the PI3K-PKB-mTOR pathway by dephosphorylating PIP3, and are classified by the position of the phosphate group they remove.

There are 10 mammalian 5-inositol phosphatases known to date, two of which are the SH-domain containing inositol phosphatases, SHIP1 and SHIP2.

In this review we will focus our attention on PTPs that counteract the three major signaling pathways commonly associated with haematological cancers, i.e. the Ras-Raf- ERK1/2, the Jak-STAT and the PI3K-PKB-mTOR pathway (Figure 1). The biochemical properties of these phosphatases and their potential role in haematological malignancies will be discussed.

Table 1. Classification of phosphatases.

	Family	Class	Sub-Class (members)	Discussed in this review
PP	PTP	Class I (Classic)	RPTP (21)	CD45
			NRPTP (17)	PTP1B, TCPTP SHP1, SHP2 HePTP
		Class I Dual specificity phosphatase (DUSP)	MKPs (11)	DUSP-1/MKP1, DUSP2/PAC-1, DUSP16/MKP-7
			Atypical DUSPs (19)	
			Slingshots (3)	
			PRLs (3)	
			CDC14s (4)	
			Myotubularins (16)	
			PTENs (5)	PTEN
		Class II	LMWPTP (1)	LMWPTP
		Class III	CDC25 (3)	
		Asp-based PTPs	EyA (4)	
	STP	PPP	PP1	
			PP2A	
			PP2B	
			PP4	
			PP5	
			PP6	
			PP7	
		PPM	PP2B	
IP	IP4P			
	IP5P			SHIP1, SHIP2

PP: protein phosphatase; IP: inositol phosphatase; PTP: protein tyrosine phosphatase; STP: serine/threonine phosphatase. STPs are subdivided into monomeric Mg^{2+}-dependent phosphatases (PPM) and a larger class of phosphatases (PPP) whose substrate specificity is determined by the formation multimeric holoenzyme complexes. PTPs are subdivided into Class I, II, III and Asp-based PTPs. Class I PTPs consists of RPTP: receptor PTP and NRPTP: nonreceptor PTPs. The IPs are classified based on the position of the inositol ring which they dephosphorylate; IP4P: inositol polyphosphate 4-phosphatase; IP5P: inositol polyphosphate 4-phosphatase.

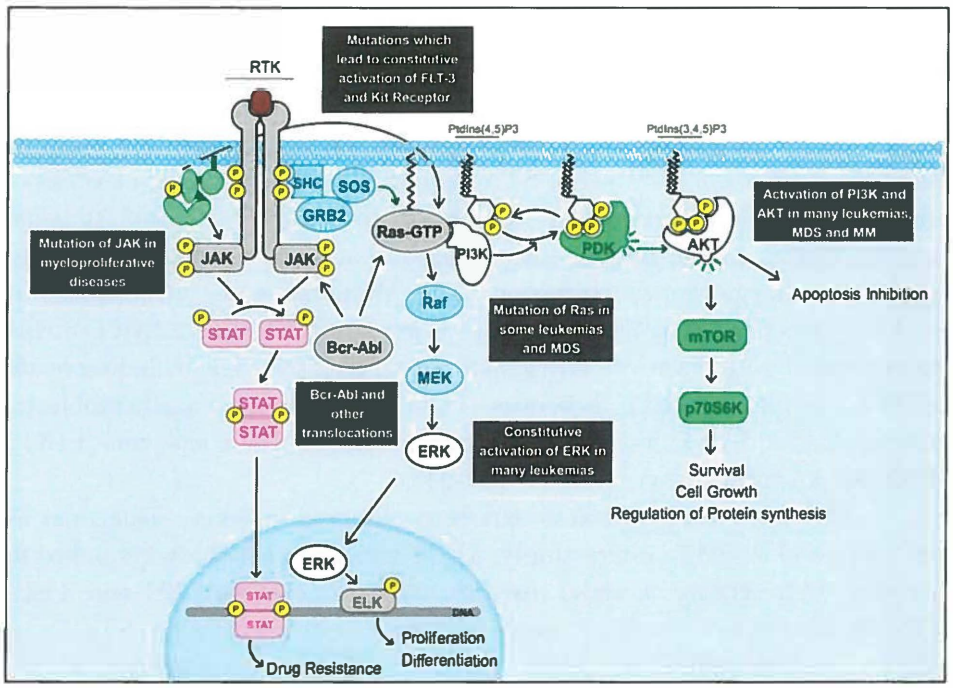

Figure 1. The three major pathways dysregulated in haematological malignancies. The Janus kinase (JAK)- signal transducer and activator of transcription (STAT), Ras- Raf-MEK-extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-OH kinase (PI3K)-protein kinase B (PKB/AKT)-mammalian target of Rapamycin (mTOR) pathways are often deregulated in blood disease. The nature of their deregulation is indicated in black boxes. Proteins that are constitutively active as a result of gene mutations are indicated in grey. RTK: Receptor Tyrosine kinase; MDS: Myelodysplastic syndrome; AKT; Protein kinase B (PKB/AKT); PDK; phosphoinositide-dependent protein kinase-1.

3. JAK-STAT SIGNALING

There are 4 members of the janus family of tyrosine kinases (JAKs); Jak1, Jak2, Jak3, and Tyk2. Cytokine stimulation of cells results in receptor dimerization, bringing their associated JAKs into close proximity to each other and allowing JAK cross- phosphorylation. The subsequent activation of JAKs enables their phosphorylation of target proteins, which include the receptor itself and their major substrates; STATs. Cytoplasmatic STATs, of which 7 family members are known in mammals, are activated upon tyrosine phosphorylation, which lead to their dimerization, nuclear translocation and regulation of target genes expression [21]. Mutations and translocations in the *JAK* genes (e.g. JAK2V617F) leading to constitutively active JAK proteins are associated with a variety of haematopoietic malignancies, including acute myeloid and lymphoblastic leukemias (JAK1, JAK2), acute megakaryoblastic leukemia (JAK2, JAK3), T-cell precursor acute lymphoblastic leukemia (JAK1), MPD and polycythemia vera (JAK2) [22-24].

Aberrant STAT activation has been observed in several leukemias as well (reviewed in [25]). Interestingly, STAT activation is not always linked to increased JAK activity in these diseases; the oncogenes Bcr-Abl and TEL-PDGFR, as well as c-src have been described to activate STAT1, STAT3 or STAT5 independently of JAKs [26-27]. Careful modulation of the JAK/STAT pathway is essential for normal cell homeostasis, and can be regulated negatively or positively by protein phosphatases depending on the PTP involved, intensity levels of the pathways and cellular context. CD45, SHP-1, SHP-2, PTP1B, TC-PTP and LMWPTP [22, 28-29] have all been demonstrated to modulate JAK/STAT pathways, and will be discussed below.

3.1 CD45

3.1.1 Characteristics and signaling

CD45 (otherwise known as Leukocyte Common Antigen) is a transmembrane tyrosine phosphatase expressed on most haematopoietic cells and their precursors except for mature erythrocytes and platelets'[30]. The intracellular tail of CD45 contains tandem repeats of two potential phosphatase domains, only one of which seems to be active (for review see [31]. CD45 is a critical positive regulator of T cell antigen receptor (TCR)- and B cell antigen receptor (BCR)-mediated signaling required for the activation and development of lymphocytes. In humans, loss of CD45 in

humans leads to severe combined immunodeficiency disease [32-34]. Cells isolated from CD45-deficient mice show decreased B-cell differentiation and accumulation of pro-B-cells, which correlates with hyperactivation of JAK1 and JAK3 upon cytokine stimulation [35]. The negative regulatory role of CD45 on JAK/STAT signaling originates either from direct association and dephosphorylation of JAK1 or JAK2, or from an alternative mechanism that involves the recruitment of the adaptor protein DOK-1 [36-37]. Loss of CD45 was also shown to increase Interleukin (IL)-3-mediated JAK2 activation and proliferation in mast cell lines, and EPO-dependent formation of erythroid burst-forming units (BFU-E colonies) [36], suggesting that CD45 activity drives differentiation in favour of proliferation.

Other proteins identified as molecular targets of CD45 are the Src Family Kinase (SFKs) members [38]. CD45 can dephosphorylate both the kinase domain and the COOH-terminal tyrosine SFKs (Figure 2). Dephosphorylation of the positive regulatory site on some SFKs leading to their inactivation was shown for Hck and Lyn during integrin mediated adhesion in macrophages [39], p56*lck* kinase activity in developing thymocytes [40] and Lyn in B cells [41]. However, dephosphorylation of COOH-terminal tyrosine, opens up the Src kinase to (auto)phosphorylation on activating residues. This appears to be the most commonly observed role for CD45 in haematopoietic cytokine signaling, providing a rationale for the positive regulatory function of CD45 in antigen receptor signaling [35], and might explain why activating CD45 mutations can result in lymphoproliferation and autoimmune disease [42]. Our group has shown that exclusion of CD45 from lipid raft signaling complexes prevents negative regulation of SFKs by CD45, providing a possible explanation for the dual positive and negative regulation of SFK activity by this phosphatase [43].

3.1.2 Role in malignancy

Different splice variants of CD45 are expressed in haematological cells during different stages of their differentiation. The low molecular weight isoform CD45RO seems to be associated with maturing blood cells, and is expressed on memory T-cells, maturing myelocytes and activated B-cells en route to becoming plasma cells [44-45]. High molecular weight isoforms, CD45RAB, CD45RBC, CD45RABC and CD45RB, are variably expressed on naive T-cells and pre-B-cells [46]. Relatively little is known about the different roles of these isotypes, although differences in cellular functioning have been shown in T-cells recombinantly expressing different CD45 isoforms

[47]. In haematological malignancy, isotype expression is often de-regulated, as was shown for AML [44, 48] and Myeloma (MM) [49-50]. However, whether this reflects differences in signaling or variation in differentiation status of cells corresponding to FAB classification remains to be determined.

CD45 deficiency in malignant T-cells was shown to result in resistance to apoptosis [51-52], whereas overexpression of CD45 in growth factor–dependent cell Lines frequently causes growth arrest and/or cell death [30]. It would therefore stand to reason that tumor cells might down-regulate CD45 expression in order to escape apoptotic processes. However, although loss of CD45 expression has been reported in patients with Hodgkin's lymphoma and in >10% of patients with paediatric Acute Lymphoblastic Leukemia (ALL), CD45 is still widely expressed in various types of hematological malignancies, and high expression levels of CD45 correlate with poor prognosis in AML and childhood ALL [53-56]. Interestingly, recent studies suggest that targeting CD45 with specific antibodies might improve survival of AML bearing mice by increasing chemotherapy uptake [57].

CD45 has been shown to play an important and complicated role in Multiple Myeloma. During normal B-cell lineage differentiation CD45 expression gradually declines, but is not lost, resulting in CD45+/dim plasma cells. In both normal and MM bone marrow CD45bright and CD45+/dim plasma cell fractions can be identified, although the CD45+/dim fraction is significantly bigger in MM patients [58], and seems to increase during progression of the disease to more malignant stages [59]. In 30% of patients, CD45 expression is completely annihilated on blood or bone marrow plasma cells, representing end stage disease and correlating with poor prognosis [60-61]. As CD45 is a negative regulator of the JAK-STAT pathway, loss of CD45 expression might partly explain the constitutive activation of this pathway observed in MM patients [62].

Several studies have investigated the proliferative capacity of the different B-and plasma cell fractions in normal and MM bone marrow and blood, and have shown that the small CD45bright compartment has a significantly higher cell proliferation index than the CD45-/dim fractions [50, 63]. CD45 expression plays a crucial role in determining signaling and proliferation of human myeloma cell responses to IL-6, insulin growth factor (IGF)-1 and other growth factors. It has repeatedly been shown that CD45+ MM cells are more responsive to IL-6 stimulation, leading to increased proliferation in this plasma cell fraction [64-65]. This effect is most likely due to an increased IL-6-induced Src kinase activation in CD45+ cells [66-67]. Interestingly, depending on cellular context, expression of CD45 can also

make cells more susceptible to apoptosis signals, which might be due to the dual role of CD45 in SFK activity or the decreased expression of the anti-apoptotic factor Bcl2 observed in CD45+ cells [58, 68]. Another explanation for this phenomenon was presented by Descamps et al, who show that IGF-1-induced activation of the PI3K/PKB survival pathway is decreased in CD45+ cells, possibly as a direct result of IGF-R dephosphorylation by CD45 [69].

CD45 expressing MM cells appear to be of a more immature subtype compared to their CD45-negative counterparts as determined by co-expression of other cell surface markers [50, 70]. In agreement with this, Matsui and co-workers showed that clonogenic capacity was contained within a CD45+ fraction of MM plasma cells, thus displaying potential progenitor cell characteristics [71]. In contrast, others showed that clonogenic potential was limited to CD45- MM cells [72]. This apparent discrepancy might be explained by differences in experimental model potentially leading to the exposure of cells to different cytokines, as it has recently been demonstrated that IL-6 induced colony growth is mostly apparent in CD45+ MM cell lines, whereas IGF-1-induced colony formation was restricted to CD45- cells [73].

In summary, the role of CD45 in haematological malignancies is a complicated one; expression of CD45 is associated with poor prognosis in AML, possibly as a result of increased SFK activity, and CD45 might potentially be used as a target for treatment in AML. In contrast, loss of CD45 expression is a poor prognostic factor in MM. The increased activity of the PI3K/PKB pathway in CD45- cells, in combination with the increased JAK-STAT activity observed in these patients, presents an opportunity for the use of kinase inhibitors of these pathways in MM treatment, whereas proliferation of CD45+ cells might be targeted by SFK or CD45 phosphatase inhibitors.

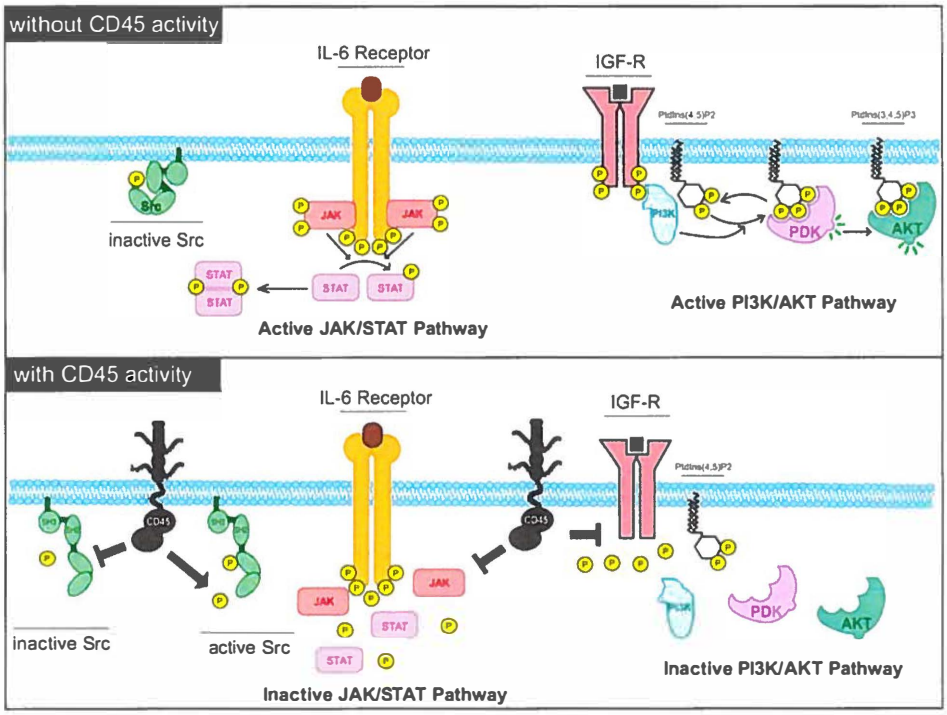

Figure 2. CD45 signaling in haematopoietic cells. Upper panel: cytokine or growth factor (e.g. Interleukin-6 [IL-6] or insulin like growth factor-1 [IGF-1]) stimulation results in dimerization of the corresponding receptor, bringing their associated JAKs into close proximity to eachother and enabling STAT activation. In addition, PI3K activety leads to formation of PtdIns(3,4,5)P3, which subsequently activates PDK1 and PKB/AKT. Lower panel: one the targets for CD45 is the Src family of kinases, represented here as Src. CD45 can dephosphorylate Src at its COOH terminal tyrosine, allowing subsequent phosphorylation and activation by cytokine stimulation, providing a positive regulation of Src kinase activity. Alternatively, CD45 can dephosphorylate Scr at its positive regulatory sequence, leaving it inactive. In addition, CD45 can negatively regulate JAK/STAT and PI3K/PKB signaling, by dephosphorylating JAK and growth factor receptors, respectively.

3.2 PTP1B

3.2.1 Characteristics and signaling

The ubiquitously expressed Protein Tyrosine Phosphatase 1B (PTP1B) is located on the cytosolic face of the endoplasmic reticulum (ER) due to a hydrophobic sequence at its C-terminal end. PTP1B is involved in multiple normal and malignant signaling pathways [3] by regulating RTKs, including epidermal growth factor receptor (EGFR) [74], PDGFR [75],

insulin receptor [76] and IGF-1 [77]. Dephosphorylation of RTKs by PTP1B occurs after endocytosis and translocation of the RTK to the ER, where after they are recycled to the cell membrane or directed to lysosomes [78]. PTP1B is thus able to dephosphorylate RTKs from different cell compartments, despite its localization to the ER. In addition to RTKs, PTP1B has other membrane-associated substrates: it can negatively regulate cytokine signaling through JAK2 and TYK2, either through dephosphorylation of STAT5a/b, or by direct association with the RTKs [79-81]. In addition, PTP1B has been shown to regulate integrin signaling through Src kinases [82].

Due to its negative regulation of multiple RTKs, PTP1B deficiency could lead to increased oncogenic signaling. Indeed, PTP1B deficient fibroblasts display increased IGF-1 receptor, EGFR, and PDGFR tyrosine phosphorylation [83]. However, Dube and co-authors reported that PTP1B can also act as a positive regulator of Ras signaling downstream of RTKs through increased p120RasGAP (Ras GTPase-activating protein) expression and p62Dok (downstream of tyrosine kinase) phosphorylation [75], which partly explains why PTP1B knockout mice do not display an increased incidence of tumors. In fact, overexpression of PTP1B has been shown in a variety of (solid) tumors, including colon cancer cells in which it activates Src kinase signaling by decreasing its negative regulatory phosphorylation [84]. In addition, overexpression of PTP1B is enough to induce breast cancer in mammary gland cells, demonstrating a role for PTP1B as oncogene in its own right [85]. As a dual role of PTP1B is arising, absence of its activity may either suppress or promote oncogenic transformation [75].

3.2.2 Role in malignancy

In humans, PTP1B is one of the negative regulators of cytokine-induced JAK2- STAT3/5 pathways [79], and mice deficient for PTP1B showed hyperphosphorylation of JAK2 [86]. Genetic ablation of PTP1B in p53 null mice decreased survival rate and increased susceptibility towards the development of B lymphomas [87], although no correlation with JAK2/STAT5 activation was observed in this study. In addition, PTP1B can negatively modulate Bcr-Abl [88], a constitutively active fusion kinase expressed as a consequence of t(9;22), the so-called Philadelphia chromosome. This oncoprotein is found in 15–30% of adults with AML, and in nearly all CML patients, where it plays a major role in the development and progression of CML through chronic, accelerated, and blast phases [89-90]. Expression of Bcr-Abl results in constitutive activation of STAT5 and alterations in signal

transduction and gene expression profiles that are associated with cytokine independence [91]. In addition to recognizing Bcr-Abl as a substrate, PTP1B disrupts the formation of a Bcr-Abl/Grb2 complex, thereby inhibiting signaling events and oncogenic transformation initiated by this oncoprotein [88]. Overexpression of PTP1B or inhibition of Bcr-Abl induced erythroid differentiation of CML cell line K562 and inhibition of PTP1B attenuates the induction of differentiation and apoptosis by STI571, a selective Bcr-Abl inhibitor, in CML cells [92]. This latter study also showed that STI571-resistant ALL cells have a significantly reduced PTP1B activity, providing strong evidence that reduction of PTP1B expression and activity leads to resistance of Bcr-Abl-positive leukemia cells to STI571.

However, increased PTP1B expression levels have been observed in activated B cell-like diffuse large B-cell lymphomas. This was associated with a decreased IL4- mediated STAT6 dephosphorylation, which is independent of Jak1, but not JAK2 [93]. Thus, whereas decreased PTP1B may increase tumorigenicity by enhancing proliferative JAK2/STAT5 signals, overexpression of PTP1B renders cancer cells unsusceptible to the anti-tumor effect of IL-4-STAT6 activation. The potential use of PTP1B in treatment of haematological malignancy may therefore completely depend on its expression level, and the signaling pathways activated in these illnesses.

3.3 TC-PTP

3.3.1 Characteristics and signaling

TC-PTP (T Cell Protein Tyrosine Phosphatase) is an intracellular enzyme encoded by *Ptpn2*. TC-PTP is mainly haematopoietic and alternative splicing of its gene allows expression of two distinct proteins, a p45 nuclear form called TC45 or TC-PTPa, and a p48 cytoplasmic form called TC48 or TC-PTP1b. TC-PTP-/- mice display defective haematopoiesis and immune function, characterized by anaemia and splenomegaly secondary to sequestration of erythrocytes and accumulation of myeloid cells [86]. In addition, TC-PTP-null mice develop severe systemic inflammation, a phenotype with potential implications in oncogenesis.

TC-PTP and PTP1B share over 70% amino acid sequence identity within their catalytic domains. However, while PTP1B is predominantly ER-localized, TC-PTP is largely nuclear. TC-PTP has been shown to control cytokine signaling events by its negative action on the JAK/STATs pathways

[94]. Both TC-PTP and PTP1B selectively recognize a motif centered on the characteristic double tyrosine residues present in the JAK activation loop, but TC-PTP exhibits specificity for JAK1 and JAK3, while PTP1B interacts with JAK2 and TYK2. TC45/TC-PTP1B is responsible for selective JAK dephosphorylation and deactivation of nuclear STAT1, STAT3, and STAT5 [95-96]. TCPTP was also shown to interact with TRAF2 downstream of the proinflammatory cytokine TNF. This interaction inactivates Src and suppresses MAPK signaling [97]. These results identify TC-PTP as a key modulator of inflammatory signals as well as lymphocyte functions.

3.3.2 Role in malignancy

Despite its clear function in normal haematopoiesis, the role of TC-PTP in oncogenic transformation remains circumspect. Similar to PTP1B, a role for reduced TC-PTP in STI571 resistance of CML cells has been established [98]. However, as for PTP1B, increased nuclear TC-PTP expression, correlating with decreased STAT6 signaling was observed in activated B-cell like lymphomas compared to other types of B-cell lymphomas and may facilitate tumor survival [99]. Bourdeau and co-authors observed that TC-PTP–deficient bone marrow stromal cells fail to support normal B lymphopoiesis due to abnormally high secretion of interferon-□, which reduced 2-fold the mitotic index of IL-7-stimulated TC-PTP-/- pre B-cells. They noted a 40% increase in apoptosis of murine early pre-B leukemic cells cultured within a TC-PTP-deficient bone marrow stroma environment [94]. Moreover, they describe constitutive phosphorylation of STAT1 in TC-PTP-/- pre-B cells, which may find practical application in the treatment of cancer. Unlike other STATs, STAT1 acts as a tumor suppressor (reviewed by [100]. Thus, reducing TC-PTP activity, thereby increasing STAT6 and STAT1 activity, may help reduce tumor burden and metastasis. TC-PTP–specific blocking agents might provide a useful pharmacologic approach toward this goal.

3.4 SHP-1

3.4.1 Characteristics and signaling, role in malignancy

Src homology 2 domain-containing phosphotyrosine phosphatases (SHP)-1 are a subfamily of cytoplasmatic non-receptor PTPs. SHP-1 (also known as PTP1C or HCP - Haematopoietic Cell Phosphatase) and SHP-2

(discussed below) are characterized by the presence of two N-terminal SH2 domains, a classic PTP domain and a C-terminal tail [101]. Their SH2 domains permit association with phosphotyrosine from proteins such as activated receptors or signaling molecules, triggering activation of the phosphatase domain and, subsequently, dephosphorylation of the substrate.

SHP-1 is mainly expressed in haematopoietic cells although it is also present in epithelial and smooth muscle cells. Via its SH2 domains, SHP-1 is recruited to c-Kit, BCR, TCR and the receptors for FCγIII, IL-3 and erythropoietin (EPO), thereby playing a role in the control of signaling cascades that couple growth factor receptors to haematopoietic cell differentiation [102-103]. Binding of SHP-1 to the EPO receptor results in dephosphorylation of the receptor-associated kinase JAK2 at position Tyr1008, thereby reducing its activity [28, 101]. Mice deficient for SHP-1 display hyperphosphorylation of JAK1 and JAK2 following Interferon-α (INF-α), Growth Hormone (GH) or EPO treatment, resulting in important immunological and haematopoietic dysfunctions [104]. In humans, the *Shp-1* gene is frequently altered in cancer cells, and various haematological malignancies, such as lymphomas, Myeloma and AML show silencing of *Shp-1* by methylation [105-107]. In addition, decreased SHP-1 mRNA and protein expression levels have been observed in diverse leukemic cell lines and paediatric AML [108-109].

SHP-1 may also affect leukemia development through its negative modulation of the Bcr-Abl pathway. Amim and co-authors (2007) observed that SHP-1 levels are markedly decreased in advanced stage CML patients compared with those in chronic phase. However, neither mutation nor DNA methylation was detected in the *Shp-1* gene in CML cell lines or patient samples, suggesting that the decrease in SHP-1 in advanced stage CML patients is due to posttranscriptional modifications [110], and implying that a decrease in SHP-1 expression levels plays a role in the progression of CML.

Together, these results suggest a potential role for SHP-1-inducing agents in cancer treatment. Interestingly, proof of concept of this hypothesis comes from experiments showing that restoration of SHP-1 expression in anaplastic large cell lymphoma using 5-aza-2'-deoxycitidine results in abrogation of constitutive JAK3- STAT3 signaling and increased drug sensitivity [111].

3.5 LMWPTP

3.5.1 Characteristics and signaling

Low molecular weight protein tyrosine phosphatases (LMWPTPs); also known as acid phosphatase locus 1 (ACP1), are a family of 18-kDa enzymes involved in cell growth regulation, cytoskeleton rearrangement and modulation of the immune response. This small molecule, which interacts with growth factor receptors and proliferation signaling pathways, possesses some interesting biochemical and structural characteristics. LMWPTP activity is regulated by phosphorylation/dephosphorylation or oxidation of the protein. It possesses two phosphorylation sites, which seem to work as switches for different functions of the phosphatase. Bucciantini et al. have shown that SFK are able to phosphorylate both sites, triggering two different effects [112]. Phosphorylation of tyrosine residue 131 increases the activity of LMWPTP 25-fold, whereas phosphorylation of tyrosine 132 leads to Grb2 recruitment. The second regulatory mechanism able to modify LMWPTP activity is the oxidation of the 12 and 17 cysteine residues, which renders the phosphatase inactive. However, the presence of an additional cysteine in the catalytic site (Cys17) makes LMWPTP able to undergo formation of an intramolecular S–S bridge. This event prevents the complete and irreversible oxidation of the catalytic cysteine (Cys12), enabling LMWPTP to rapidly recover its activity [113].

LMWPTP has been shown to be associated with molecules involved in cell growth and proliferation, such as PDGFR [114], JAK2 [115], STAT5 [29, 116], Focal adhesion kinase (FAK) [117], Ephrin A2, receptors (Eph A2) [118] and β-Catenin [119]. Although mostly regarded as negative regulator of kinase activity, a positive action of

LMWPTP in cell growth and proliferation signaling has been also described [115, 120]. LMWPTP is an important regulator of STAT5 activity during megakaryocyte differentiation. Rigacci and co-workers suggested that the association of LMWPTP with STAT5 prevents phosphorylation of this transcription factor in the absence of a growth factor stimulus [29]. In contrast, LMWPTP oxidation mediates the sustained phase of JAK2 phosphorylation, which is required for the antiapoptotic effects of IGF-I and serum on pancreatic cells [115]. This suggests that inhibition of LMWPTP leads to enhanced and sustained phosphorylation of the JAK/STAT pathway and suppression of apoptosis, and as such makes this PTP an interesting candidate in IGF-1-mediated MM cell survival. However, the oxidative switch

of LMWPTP and its function is not completely understood, and it has also been observed that oxidation of this phosphatase is involved in Grb2 binding and increased ERK activation, implying that LMWPTP oxidation can directly trigger a prosurvival signal against the oxidant environment [120].

3.5.2 Role in malignancy

Increased expression levels of LMWPTP have been reported in several human tumors, such as neuroblastoma, breast and colon cancer. Analysis of patient survival indicates that higher LMWPTP levels are predictive of an unfavourable outcome, meaning that LMWPTP could confer tumor aggressiveness and its expression might be used as a marker in some types of cancer [121].

Interestingly, LMWPTP has been pointed out together with ZAP70 as predictors for treatment requirement in a case reported by Chen et al 2005. These authors have shown that a patient with chronic lymphocytic leukemia presented a gradual loss of LMWPTP and ZAP70 as well as the active form of ZAP70 (phospho-ZAP70 tyr492) during continued chemotherapy. In addition, the return of these proteins to normal levels correlated with requirement for treatment [122]. Therefore, although LMWPTP has a negative regulatory function in JAK/STAT activation and hence would appear to be tumor suppressive, its overexpression in many cancers suggests that this does not hold true for transformed cells, and may make this phosphatase a target for therapy in haematological malignancies. However, the regulation of its switches (phosphorylation and oxidation) and state (activated and inhibited) and their importance for the normal and cancer cell biology require further study.

4. RAS-RAF-MEK-ERK SIGNALING.

The Ras signaling pathway has been extensively studied and reviewed. Upon cytokine signaling, the adaptor protein Grb2, which is complexed to the guanine nucleotide exchange factor (GEF) Son of Sevenless (Sos), is recruited to activated receptors through its SH2 domain. The subsequent membrane localisation of this GEF allows activation of the membrane-bound small GTPase Ras by facilitating its ADP release and subsequent ATP binding. Ras activation results in a cascade of mitogen-activated protein kinase (MAPK) activation, where MAPK kinase kinase (e.g., Raf) activates MAPK kinase (e.g., MEK1/2), which subsequently phosphorylates MAPK (e.g., extracellular signal regulated kinase; ERK1/2) [123]. MAPKs are subdivided into three subfamilies based on sequence similarity, differential regulation and substrate specificity: ERK1/2, c-Jun N-Terminal Kinase (JNK) (JNK 1,2 and 3) and p38 (α,β,δ and γ) [124].

Activating Ras mutations, predominantly investigated in leukemias and MDS, have been observed in a large cohort of studies (Reviewed in [125]. Ras activity and function depends on its membrane localisation, a process facilitated by farnesylation of the protein, and farnesyl transferase inhibitors have therefore been extensively tested in tumor cell biology. However, clinical phase II trials with these inhibitors were only marginally successful, and despite achieving clinically relevant positive results in some studies, this did not seem to be correlated to RAS mutation status of patients [126-128]. Downstream of Ras, mutations in Raf and ERK1/2 have been observed in a number of haematological malignancies (reviewed in [129]). Increased phosphorylation of ERK1/2 is frequent in leukemia, and appears to play an important role in drug resistance [130]. In contrast, in low risk MDS patients, a decreased ERK1/2 activity was observed in neutrophils and $CD34^+$ progenitor cells, which correlated with decreased cell functionality [131-133]. As such, phosphatases interfering in MAPK signaling may be of functional consequence in haematological disease and may provide a target for treatment. Some such phosphatases include SHP-2, HePTP, and the MAPK phosphatases (MKPs), and these will be further discussed below.

4.1 SHP-2

4.1.1 Characteristics and signaling

The protein tyrosine phosphatase SHP-2 is encoded by the PTPN11 gene (proteintyrosine phosphatase non-receptor-type 11) and localized on 12q24 [134]. SHP-2 is expressed ubiquitously, but particularly strongly in blood cells. In its basal state, SHP-2 activity is suppressed by intramolecular interactions between residues in the "backsideloop" of the N-SH2 domain (the side opposite the phosphotyrosyl peptide binding pocket) and the catalytic surface of the PTP domain [135]. Upon growth factor or cytokine stimulation, SHP-2 is recruited via its SH2 domains to phosphorylated tyrosine residues on RTKs (e.g. Kit-ligand, IL-3, EPO and granulocyte-monocyte colonystimulating factor (GMCSF)) and/or scaffolding adaptors, such as insulin receptor substrate, fibroblast growth factor receptor substrate, or Grb2-associated binder (GAB) proteins [136]. Phosphotyrosyl peptide binding to the N-SH2 domain disrupts the autoinhibitory interface; leading to exposure of the PTP domain and catalytic activation [7, 135]. Activation of SHP-2 generally leads to activation of the Ras/Ras/ERK pathway, but its precise target(s) remains controversial. SHP-2 activates SFKs evoked by RTK and integrin signaling in fibroblasts, and SFKs in turn are required for sustained Ras activation on endomembranes [137].

Other authors suggest that SHP-2 ensures Ras signaling by dephosphorylating potential recruitment sites for p120RasGAP, a GTPase activating protein that promotes Ras inactivation [138]. SHP-2 is essential for myeloid differentiation [83], and can negatively modulate c-Kit signaling by interacting with specific phosphotyrosine residues on this receptor [103]. Kit signaling is important in erythropoiesis, lymphopoiesis, megakaryopoiesis and mast cell development and function, and its expression and constitutive activation is found in for instance mast cell leukemia and AML [139].

4.1.2 Role in malignancy

SHP-2 was the first PTP implicated in leukemogenesis. Somatic gain of function mutations in the PTPN11 gene are the most common cause of sporadic juvenile myelomonocytic leukemia (JMML), implicated in about 35% of cases [140]. In addition, PTPN11 mutations occur at lower incidence in other myeloid neoplasms such AML (~5%), chronic myelomonocytic leukemia (CMML), and myelodysplastic syndrome (MDS) [140-141], as well as in the

most common form of childhood leukemia, B-acute lymphoblastic leukemia (B-ALL) (~10%) [7, 96]. Germline mutations in the SHP-2 gene occur in 50% of individuals with Nooman syndrome (NS), a developmental disorder with short stature, facial dysmorphia, skeletal anomalies and heart defects [141]. Children with this syndrome have a growth disorder as well as a predisposition to develop JMML [142]. Activating PTPN11 mutations negatively regulate the cellular response to Growth Hormone (GH) and explain the growth disorder observed in these patients. *In vitro* experiments demonstrated that SHP-2 associates directly with the GH receptor (GHR) in response to GH [143] and acts as a cytosolic phosphatase of STAT5, downregulating its activity [144]. In addition, a decreased STAT3 activation has been observed in NS/JMML cells with PTPN1 mutation [145]. As discussed above, SHP-2 is required for full activation of the Ras/Raf/Erk pathway and for multiple receptor-evoked functions, including cell proliferation, differentiation, and migration [101]. Indeed, gain of function mutations in PTPN11 have been shown to result in hyperactivation of ERK and protein kinase B activation [146-147]. Thus, the predisposition of the Noonan Syndrome patients to JMML is related to the activation of Ras/MAPK pathway by SHP-2 activation. It is important to emphasize that mutations in the RAS family members KRAS or NRAS genes, PTPN11 gene, or the RAS-GAP NF1 are observed in 65%–85% of JMML patients [148].

Konieczna and co-authors (2008) found that constitutive activation of SHP-2 in mice cooperates with progression of myeloproliferative disorders. They showed that Interferon Consensus Sequence Binding Protein (ICSBP, also known as IRF8), an interferon-regulatory transcription factor, functions as a leukemia tumor suppressor only when phosphorylated. Coexpression of a constitutively active form of SHP-2 synergized with ICSBP haploinsufficiency to accelerate progression to AML, induce cytokine hypersensitivity and apoptosis resistance in vivo [134].

SHP-2 can also function as a positive factor in IL-6 signaling by stabilizing JAK2 or inducing Src kinase activation [149]. IL-6-induced JAK1,2-STAT3 signaling is one of the major growth signals in Myeloma cells [62]. It has recently been shown that c-MET enhances IL-6-induced proliferation of MM cells through increased activation of SHP2, suggesting a possible role of SHP-2 in MM pathology [150].

In conclusion, the evidence to date suggests that the role of SHP-2 in leukemogenesis is more prominent than its role in tumor suppression, implying a potential role for SHP-2 inhibitors as a molecular target of therapy. Interestingly, this seems to be corroborated by the finding that increased

expression of SHP-2, which was correlated with enhanced FLT3 signaling in AML patients, was reduced by treatment of AML cells with a c-KIT/FLT3 kinase inhibitor [151].

4.2 HePTP

4.2.1 Characteristics and signaling, role in malignancy

Haematopoietic PTP (HePTP), also named leukocyte PTP (LC-PTP), is a NRPTP that contains a kinase interacting motif, allowing it to bind to ERK and the MAPK p38 [152]. This results in conformational changes in both the PTP and its substrate, leading to dephosphorylation of the substrate, and subsequent phosphorylation of HePTP by the bound kinase [153]. In T-cells, HePTP functions as an early response gene, regulating phosphorylation state during IL-2-induced proliferation [154]. HePTP overexpression results in decreased ERK phosphorylation upon TCR signaling [155], whereas its reduction increased PMA-induced ERK1/2 activity in spleen cells from HePTP knock out mice [156]. In its capacity as a modulator of ERK1/2 activity and nuclear translocation, HePTP is also involved in megakaryocyte differentiation [157]. In addition, HePTP can be phosphorylated by protein kinase A in B-cells, where it inhibits p38 induction by betaadrenergic receptor agonist signaling [158].

Expression of HePTP is limited to tissues of haematological origin such as thymus, spleen and all myeloid and lymphoid cells. As such, it is interesting to note that the HePTP gene is mapped next to another haematopoietic specific PTP, CD45, on chromosome 1q32 [159] and might therefore be generated by gene duplication of a common ancestral gene [160]. Deletions of this chromosomal region have been frequently found in B-cell lymphomas, which corresponds with a significantly decreased protein expression of HePTP observed in paediatric B-cell lymphoma cases [161-162]. In contrast, triplication of the HePTP gene and protein overexpression was observed in MDS and AML patients, suggesting a cell-type specific role for aberrant HePTP expression in haematological malignancies [163]. However, further studies will have to correlate changes in HePTP expression to downstream signaling strength in haematological malignancies, and determine whether this promising candidate phosphatase may be a potential target for treatment in haematological tumors.

4.3 MKPs

4.3.1 Characteristics and signaling

MAPKs transduce extracellular signals from environmental stress hormones, growth factors and cytokines which results in control of diverse physiological processes such as proliferation, differentiation, migration and apoptosis (reviewed in [17, 164]). MAPK phosphatases (MKPs), a family of DUSPs, are able to inactivate MAPKs through dephosphorylation of tyrosine and/or threonine residues in the activation loop of MAPKs. Based on sequence similarity, substrate specificity, gene structure and subcellular localization the ten MKPs can be classified into three subfamilies. The first group is composed of nuclear MKPs which include DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5/hVH-3. The second group of cytoplasmic MKPs comprises DUSP6/MKP-3, DUSP7/MKPX and DUSP9/MKP-4, and the final group consists of DUSP8/hVH-5, DUSP10/MKP-5 and DUSP16/MKP-7, which are found both in the cytoplasm and the nucleus. Abnormalities in MAPK pathways have been associated with a wide variety of human diseases, including cancer (reviewed in [17]). The role of ERK1/2 in haematological malignancies is well documented, whereas the involvement of the JNK and p38 MAPKs is more circumspect [129]. Activation of ERK is usually associated with cell proliferation and survival, whereas JNK and p38 contribute to cell apoptosis in response to stress [165]. The balance between MAPK and MKP activity directs cell fate to either survival or apoptosis. Negative regulators of ERK, such as MKP DUSP2/PAC1, are excellent candidate tumor suppressors. However, other MKPs, like MKP-1, have been associated with survival and tumorigenesis and are overexpressed in many types of cancer [165]. It is interesting that different DUSPs can have either a positive or negative regulatory role in ERK activation, as increased ERK pathway activation is observed in 30 to 50% of AML cases, whereas some low-risk MDS patients show decreased ERK phosphorylation [132, 166].

4.3.2 Role in malignancy

Recently, the DUSP16/MKP-7 was shown to be associated with leukemia. Altered subnuclear localization of the transcription factor Runx1, responsible for normal haematopoiesis, was linked to the etiology of AML by upregulating microRNA-24 (miR-24). miR-24 expression leads to inhibition of MKP-7 expression, consequently enhancing myeloid cell proliferation and

blocking granulocyte differentiation by increasing p38 phosphorylation [167]. Although this may seem somewhat counterintuitive, as p38 activity has been associated with cell death and reduced tumorigenesis [168], p38 has also been shown to be detrimental for granulocyte development and others have demonstrated that decreasing constitutive p38 activation may restore normal hematopoiesis in malignant cells [169-170].

The MKP DUSP2/PAC-1 is predominantly expressed in haematopoietic tissue, and acts as a negative regulator of MAPK signaling [171-173]. Loss of DUSP2/PAC-1 expression has been associated with elevated levels of ERK activation in acute leukemia, as Kim and co-authors showed that increased expression and activity of ERK and MEK in 17 of 26 cases (65.4%) indeed corresponded to downregulation of DUSP2/PAC-1 [174]. Thus, downregulation of MKPs can be responsible for the increased MAPK activation that is often described to contribute to haematological malignancy. Increasing these MKPs' activity for treatment of these diseases remains a challenge.

However, enhanced MKP activity has also been linked to haematological illness. Constitutive overexpression of two transcripts related to DUSP2/PAC-1 was observed in large granular lymphocyte (LGL) leukemia [175]. LGL leukemia is a lymphoproliferative disorder originating either from mature T cells or natural killer (NK) cells, and often associated with autoimmune disease (reviewed in [176]. The physiological role of DUSP2/PAC-1 in the immune system was described by Jeffrey et al (2006) who showed that PAC-1-null mice display a decreased expression of proinflammatory mediators and cytokines such as IL-6, IL12-α, cyclooxygenase-2 and IL-1β in response to LPS stimulation [173]. As the etiology of LGL leukemia is not completely elucidated, the discovery of DUSP2/PAC-1 overexpression in this disease has provided a focus in the search for a therapeutic target.

Another MAPK phosphatase with a possible role in haematological cancer is the DUSP1/MKP-1, expression of which is induced by growth factors and stress signals in human cells [177]. Initially, MKP-1 was shown to dephosphorylate ERK and negatively modulate cell proliferation [178]. However, recent studies have described an antiapoptotic effect of MKP-1 by negatively modulating JNK and p38 [179]. In addition, enhanced activity of the RAF/MEK/ERK pathway can be associated with increased mRNA levels of DUSP1/MKP-1 as shown in a study from Staber and co-workers, who demonstrated overexpression of MKP-1 accompanied by RAF/MEK/ERK pathway upregulation in bone marrow of relapsed AML patients compared to

AML samples before high-dose chemotherapy [180]. This positive regulatory role for MKP-1 in cell survival was later confirmed by others, showing that MKP-1 depletion in breast cancer induces apoptosis [165].

Together, these data indicate that MPKs can exert both positive and negative actions on leukemogenesis, and suggest that that they might be viable targets for interference. Especially downregulation of MKP-1 would seem a promising avenue of investigation in cancer treatment. However, more research into the exact mechanisms of the different MKPs is clearly needed.

5. PI3K-PKB SIGNALING

PI3K signaling is initiated upon growth factor or chemokine receptor activation, either through direct recruitment of the SH2 domain containing p85 subunit of PI3K to the receptors or through binding of the kinase to active Ras GTPase. Once translocated from cytosol to plasma membrane, this lipid kinase phosphorylates membrane anchored phosphoinositides on the 3-position of the inositol ring. The main products formed are PtdIns(3,4)P2 and PtdIns(3,4,5)P3, which act as second messengers by recruiting molecules with a phospholipid binding (PH) domain to the membrane, where they are subsequently activated. One of these is protein kinase B (PKB, also known as Akt), which plays a major role in cell survival and phosphorylates, amongst others, mTOR, GSK3 and the forkhead transcription factors. Many tumors, including blood cancers, present with constitutive activation of the PI3K-PKB-mTOR pathway (reviewed in [181-182]). Although activating mutations in the PI3K gene (PIK3CA) and PKB gene (AKT E17K) have been described, their occurrence seems to be rare in haematological malignancies [183-184]. More often, constitutive PI3K signaling is thought to be a secondary effect: the presence of activating mutations in receptor tyrosine kinases or Ras, oncogenic translocation products Bcr-Abl and Tel-Abl, as well as autocrine cytokine signaling loops have all been cited as contributing factors [185]. Regardless of the activating mechanism, targeting this pathway with inhibitors of either PI3K/PKB or mTOR appears to be a promising approach in the treatment of leukemias (reviewed in [186]). Phosphatases counteracting the PI3K-initiated signaling cascade include PTEN, SHIP and SHIP-2. Their role in normal cell physiology and leukemic transformation will be discussed below.

5.1 PTEN

5.1.1 Characteristics and signaling

Phosphatase and tensin homolog (PTEN), located on 10q23.3, is a dual-specificity phosphatase that has both lipid and protein phosphatase activity. PTEN classically converts PtdIns(3,4,5)P3 at the plasma membrane to PtdIns(4,5)P2, thereby directly antagonizing the activity of PI3K [187]. Therefore, PTEN inactivation results in constitutive activation of the PI3K/PKB pathway and subsequent increases in protein synthesis, cell cycle progression, migration and survival [188].

Although several studies where PTEN was overexpressed or analysed in tumorigenic cell lines and tissues have shown that PTEN was mostly localized in the cytoplasm, studies in healthy tissues have suggested that PTEN is originally localized in the nucleus [189-191]. Loss of nuclear PTEN has now been observed in a variety of tumors, suggesting an association between a lack of nuclear PTEN and mitotic index [192-194]. This seems to be corroborated by the fact that nuclear presence of PTEN is apparent in quiescent cells, but in actively dividing cells PTEN localizes mostly in the cytosol [195].

5.1.2 Role in malignancy

Deletion of *Pten* in mouse haematopoietic stem cells leads to a myeloproliferative disorder which is followed by acute T-ALL [196]. In humans, many somatic mutations have been described for the PTEN gene, although these occur in the minority of the tumors. Until recently, PTEN mutations had only been described sporadically in leukemia and lymphomas. However, Gutierrez and collaborators have now identified abnormalities in the PTEN gene with a frequency of 8.7% in T-cell acute lymphoblastic leukemia (T-ALL) patients, including homozygous and heterozygous deletions [197].

These inactivating mutations carry a negative prognostic risk, related to a high risk of treatment failure [198]. This statement is in agreement with Palomero and co-workers who have reported the association of PTEN mutations and non-responsiveness to γ- secretase inhibitors (GSI). GSI target the NOTCH1 signaling pathway, which is present in the majority of T-ALL cases, and was shown to reduce PTEN expression via HES1. This in turn leads to a constitutive PKB activation which renders the cells resistant to GSI [199].

Loss of functional PTEN has been attributed to PTEN promotor hypermethylation in a number of cancers. Recently, *Pten* epigenetic mutations have been also described in JMML. Liu et al suggested that DNA hypermethylation in the 5' upstream region of the PTEN gene was responsible for deficient PTEN gene expression in 77% of the patients analysed [200]. In addition, reduced PTEN expression in ALL due to promoter methylation resulted in imatinib resistance in ALL cell lines [201]. However, these data were disputed by Batz et al 2009, who showed that hypermethylation of the PTEN upstream CpG island in JMML is infrequent and probably not a relevant epigenetic mechanism of disordered PTEN expression in leukemia

[202]. Many reasons could account for this difference such as geographic variation or other heterogeneity between the two patient cohorts.

Other regulatory mechanisms for PTEN expression have been described. For instance, down modulation of PTEN expression, resulting in constitutive PKB activation in leukemia, has been attributed to the increased expression of oncogenic microRNA miR-21 and miR-155 in these tumor cells [203]. Antisense oligonucleotides targeting these miRNAs restored PTEN expression and enhanced apoptotic activity. Aberrant microRNA expression has been described in a number of malignancies, and strategies to target these miRNAs for the treatment of cancer are under investigation [204].

Low PTEN protein expression has been observed in AML, ALL, and paediatric B-ALL by flow cytometry, but surprisingly, increased expression was observed in monocytic leukemia [205]. In addition, using immunohistochemistry, Gauffiun et al. found protein overexpression of PTEN in paediatric ALL samples [109]. These conflicting data might be explained by Sylva and co-workers who reported increased PTEN protein levels in T-ALL cells, but found that this was correlated with decreased PTEN activity as a result of increased casein kinase 2 (CK2) activity and reactive oxygen species (ROS) production in these cells [206]. Inhibiting CK2 activity and ROS production restored PTEN activity, and reduced PI3K/PKB signaling in this study. PTEN is also inactivated through phosphorylation on serine/threonine residues at its C-terminus, and phosphorylated PTEN levels are associated with poor prognosis in AML [207-208].

It has been suggested that resistance to the apoptosis inducing agent TRAIL was due to constitutive PTEN phosphorylation in ALL cell lines [209]. As CK2 is the main kinase responsible for PTEN phosphorylation, there might be a potential role for CK2 inhibitors in the treatment of haematological malignancies presenting with PTEN phosphorylation. In fact, the use of CK2 as therapeutic target in AML was suggested after its expression was found to be an unfavourable prognostic factor in these leukemias, although unfortunately PTEN phosphorylation status was not investigated in this study [210].

5.2 SHIP1/2

5.2.1 Characteristics and signaling

Although PTEN is classified as PTP, most of its actions in cancer are derived from its major role as a lipid phosphatase. Other lipid phosphatases have been described that are important in health and disease [20]. One of these is the SH2 domain containing 5- inositol phosphatase, SHIP, which dephosphorylates inositol lipids at the 5D position of the inositol ring, hydrolysing PtdIns(3,4,5)P3 to PtdIns(3,4)P2. As such, SHIP has mostly been seen as negative regulator of PI3K activity. However, the SHIP-1 protein contains an N-terminal SH2 domain, a central conserved 5-phosphatase domain, two potential phosphotyrosine binding domains, and a C-terminal proline rich SH3 binding region [19]. These different regions ensure that besides being a lipid phosphatase, SHIP also acts as an adaptor protein by binding to other molecules, such as Src kinase and the Ras activating protein Grb2 (reviewed by [211-212]). Therefore, studies into increased and eliminated SHIP expression on cellular effects need to be interpreted with care, as they might not only reflect the effect on the PtdIns(3,4)2 levels, but also take into account other SHIP functions. For instance, expression of an IGF-R mutant resulting in improper membrane localisation of SHIP1 did not affect PKB activity, although IGF-induced ERK phosphorylation was downregulated, suggesting a PtdIns(3,4)P2-independent effect [213]. In addition, more and more evidence suggests that although PTEN is the major regulator of PI3K signaling by decreasing PtdIns(3,4,5)P3 levels, fine tuning of PI3K signaling is performed by SHIPs and not necessarily inhibitory. Although PtdIns(3,4,5)P3 is required, exogenous PtdIns(3,4)P2 enhances PKB phosphorylation, and is necessary for full PKB activation [214]. These results were further explained by Ma and colleagues, who showed that whereas PtdIns(3,4,5)P3 was responsible for Thr308 phosphorylation of PKB, PtdInd(3,4)P2 was required for Ser473 and hence membrane activity of PKB [215]. Nevertheless, the level of phosphorylation of PKB on Thr308, but not Ser473, was associated with poor prognosis in AML patients, suggesting that PtdIns(3,4,5)P3 is the major determinant of clinical outcome [216].

5.2.2 Role in malignancy

Multiple isoforms of SHIP-1 have been described, including a full length 145 kDa SHIP-1, a ~135 kDa SHIPbeta containing a deletion between

the tyrosine binding motifs [217-218], a ~108 kDa SHIPdelta which lacks 167 nucleotides in the C-terminal region [219] and a stem cell specific 104 kDa isoform s-SHIP (also termed SIP-110 in humans) which lacks the SH2 domain [220-224]. SHIP-1 and SHIP-2 share 38% amino acid homology [225-227], but whereas SHIP-2 is ubiquitously expressed, SHIP-1 is predominantly found in cells of the hematopoietic lineage and bone marrow microenvironment, with a higher expression in CD34+ progenitor cells and T-cells and a lower expression in B-cells and granulocytes [228-229]. In addition, SHIP-1 rather then SHIP2 is responsible for controlling PtdIns(3,4,5)P3 levels in hematopoietic cells [230]. SHIP-/- mice show increased haematopoietic stem cell numbers and proliferation, splenomegalomy and pulmonary macrophage infiltration [231]. Importantly, SHIP-/- mice display disturbed haematopoiesis as evidenced by decreased lymphopoiesis and increased myelopoiesis resulting in Myeloproliferative disease, suggesting that cell lineages are differentially affected by SHIP deletion [232]. Luo and co-workers demonstrated the presence of a SHIP inactivating mutation in a patient with AML. Transduction of T-cell lines with SHIP harbouring this mutation resulted in increased cytokine induced PKB phosphorylation and drug resistance [233]. This same group later reported the presence of multiple mutations in the coding region of the SHIP gene *INPP5D* in a cohort of Chinese leukemia patients [234], although a similar study in a Caucasian population showed that coding-region SHIP mutations were rare, and no differences in gene expression between AML and healthy controls were observed [235]. Very recently, an American group reported decreased SHIP RNA and protein levels in almost all primary ALL samples studied, in conjunction with inactivating mutations and alternative splicing in the SHIP gene sequence [236]. Primary CLL samples do not contain SHIP2, and variable expression of SHIP1; decreased expression and tyrosine phosphorylation of SHIP1 was observed in CLL patients that are positive for the tyrosine kinase ZAP70, a poor prognostic indicator [237]. In addition to mutations in the SHIP gene itself, SHIP might also play a role in Bcr-Abl mediated malignancies, as SHIP expression is inhibited by this oncogenic fusion kinase, and SHIP-2 binds specifically to the SH3 domain of Abl. [238-239]. As activity of SHIPs is regulated by their protein levels, these studies imply a role for decreased SHIP activity in haematological malignancies [240]. Indeed, a SHIP agonist was identified, which increased SHIP activity, decreased PKB/AKT phosphorylation and was able to induce cytotoxicity in Myeloma cells [241-242].

However, there is some controversy concerning the exact role of SHIP in tumor cell survival. A recently published report shows that loss of SHIP expression may increase leukemia incidence [243], whereas others have shown that SHIP deletion did not result in increased leukemogenesis but rather inhibited cell growth [244]. In addition, whereas overexpression of SHIP1 leads to apoptosis in murine myeloid cells, reexpression of SHIP in Jurkat T-cells, normally devoid of both SHIP and PTEN, leads to increased cell cycle time, but not apoptosis [245-246]. As PtdInd(3,4)P2 is capable of PKB/AKT activation, SHIP activity may actually contribute to PKB activation and hence lead to growth and survival of neoplastic cells. Indeed, beside PtdIns(3,4,5)P3, PtdIns(3,4)P2 levels are increased in leukemia cells and increased levels of PtdIns(3,4)P2 promote the transformation and tumorigenicity of mouse embryonic fibroblasts [247-248]. Furthermore, a recently identified specific SHIP-1 inhibitor was able to reduce leukemic and MM cell growth *in vitro*, which could be rescued by the addition of endogenous PtdInd(3,4)P2 to cultures [249]. It is therefore very likely that PKB activity and subsequent cellular effects, such as survival, are very delicately regulated by the levels of PtdIns(3,4,5)P3 and PtdIns(3,4)P2, where disruption in either direction could lead to cell death. Its clear involvement in diverse haematological malignancies and the availability of both agonists and antagonists make SHIP an interesting target candidate for treatment of blood disease.

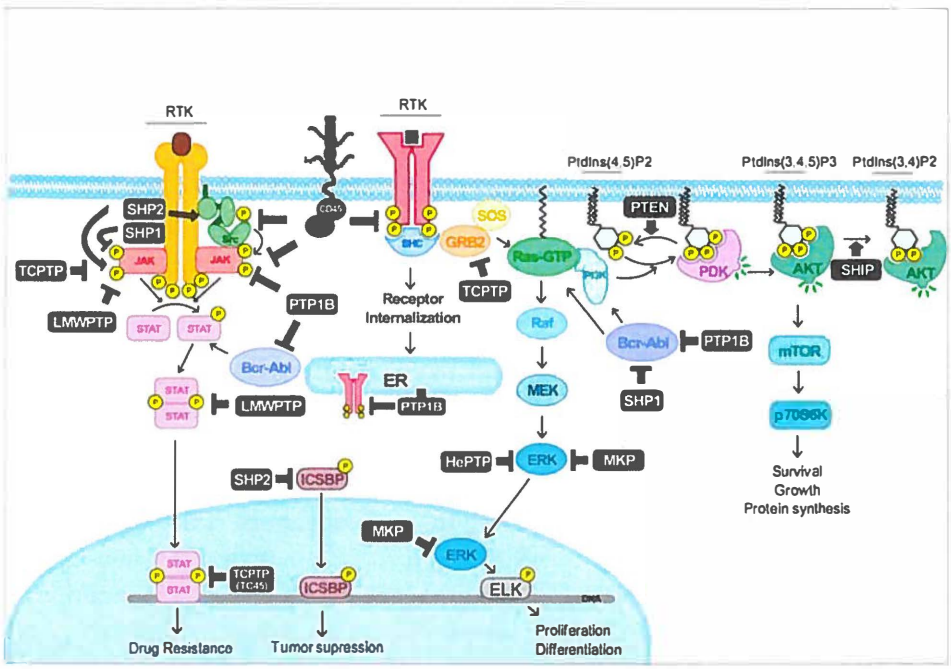

Figure 3. The role of phosphatases on the three major kinase pathways dysregulated in haematological malignancies. The JAK-STAT pathway can be upregulated by SHP2 which stabilizes JAK2. On the other hand, LMWPTP, SHP-1, PTP1B and CD45 can negatively modulate JAKs. Inhibition of Bcr-Abl by PTP1B can also negatively modulate STAT signaling. In addition, CD45 is able to directly inactivate Receptor Tyrosine Kinases (RTK) and inactivate Src, thus affecting JAK-STAT signaling. STATs can be dephosphorylated by LMWPTP in the cytosol and by TCPTP in the nucleus. RTKs can be dephosphorylated and inactivated by TCPTP in the cytosol face of Endoplasmic Reticulum (ER). In MAPK pathways, the main phosphatases involved are MAPK phosphatases (MKP). MKP can dephosphorylate ERK in the cytosol (DUSP16) or in the nucleus (DUSP2/PAC-1 and DUSP1/MKP-1). Another phosphatase able to negatively modulate ERK is HePTP. The major negative modulator of PI3K-PKB signaling is the lipid phosphatase PTEN. PTEN can dephosphorylate PtdIns(3,4,5)P3 to PtdIns(4,5)P2, thereby preventing PKB activation. PtdIns(3,4,5)P3 can also be dephosphorylation by SHIP at the position 5, although the ensuing product PtdIns(3,4)P2 has also been shown to be able to activate PKB.

6. CONCLUDING REMARKS

Most research to date has focussed on the role of kinases in cancer cell biology, and as a consequence, kinase inhibitors play a major role in novel therapeutic development. However, it is becoming increasingly clear that phosphatases are involved in numerous diseases, including haematological cancers, thus providing an additional focus of interest in drug development (Figure 3, Table 2). In fact, several specific serine/threonine and tyrosine phosphatase inhibitors are under development, some of which have already been shown to be orally tolerable [14, 250-251]. Phosphatases are generally regarded as inhibitors of oncogenic signaling and hence tumor suppressors, which might account for the lack of attention they have received in haematological cancer treatment to date. Reduction of phosphatase activity is observed in a number of malignancies and seems difficult to overcome using targeted medicine. The challenge here would be to develop compounds that activate rather than inhibit phosphatases. For SHP-1 this has already been achieved; in a search for SH2-domain inhibitors, Park et all discovered that SHP-1 is specifically activated by peptidyl aldehydes [252]. However, this approach would not work in patients where reduced phosphatase activity is a result of an inactivating mutation or deletion. Gene therapy or protein delivery systems such as TAT fusion proteins would be required to help these patients [253]. Unfortunately, such technologies are still in infancy and safety issues make them unlikely to contribute substantially towards cancer treatment in the upcoming years. In some instances, i.e. PTEN and SHIP, it has been reported that reduced phosphatase expression in leukemic cells was a result of aberrant miRNA expression [254]. As mentioned before, these miRNA are now being considered as targets for therapy [255].

Although downregulation of phosphatase activity often is apparent in hematological malignancy, there is also a growing body of evidence suggesting that overexpression of phosphatases might contribute to the tumor pathology, as is the case with for instance SHP-2, LMWPTP, PTP1B and CD45. In these cases, specific inhibitors might find clinical applications. However, as not all patients exhibit increased phosphatase activity, the use of these inhibitors calls for personalised medicine. In addition, as some phosphatases might have both positive and negative regulatory functions, extensive research is needed to discover the long-term effects of the use of these inhibitors. Due to their major role in cell growth, proliferation and survival, protein and lipid phosphatases are of great potential interest in cancer treatment, and deserve a lot more attention than they currently receive. Further research into their precise role

in normal and tumor cell biology might, in time, make them a more appreciated clinical target.

ACKNOWLEDGEMENTS

The authors would like to thank Carlos Eduardo Santoro Pedroso Filho for his technical assistance in making the figures.

Table 2. Involvement of phosphatases in normal and malignant signaling.

PTP	Normal function	Substrates	Nature of Change	Consequences
CD45	Reduced JAK/STAT Reduced/increased Src	Src, JAK [38]	1. Deletion 2. Increased expression 3. Reduced expression	1. Severe Combined Immunodeficiency Syndrome [33, 34] 2. Poor prognosis in AML and ALL [53-56] 3. Increased IGF-1, decreased IL-6 signalling in MM [68,69]
PTP1B	Reduced RTK Reduced JAK/STAT	RTKs (such as EGFR [74], PDGFR [75], Insulin Receptor [76], IGFR [77], BCR-Abl [88], JAK2, TYK2, STAT5 [244-246].	1. Increased expression 2. Reduced expression	1. Decreased anti-tumorigenic STAT6 in DLBCL [93] 2. Role in CML resistance [92]
TC-PTP	Reduced JAK/STAT	JAK1, JAK3 [247], STAT1, STAT3, STAT5 [248,249]	1. Increased expression 2. Reduced expression	1. Decreased anti-tumorigenic STAT6 in DLBCL [93] 2. Role in CML resistance [98]
SHP-1	Reduced JAK/STAT	JAK, Kit [250, 251]	1. Silencing by methylation 2. Decreased protein expression	1. Observed in leukemia, lymphoma, MM [105-107] 2. Observed in CML [110]
LMWPTP	Reduced JAK/STAT	JAK, STAT5 [252]	Increased expression	Unfavourable prognosis in solid tumours and leukemia [121-122]
SHP-2	Reduced GH signalling Enhanced Ras/MAPK Reduced JAK/STAT	JAK2, Src [149], ICSBP [134], Kit [255], STAT5 [256] GH receptor [143]	1. Constitutive active 2. Gain-of-function mutation	1. Myeloproliferative disease in mice [134] 2. Predisposition to JMML, growth disorder. Observed in AML, CMML, MDS, B-ALL [7,96,140-142]
HePTP	Proliferation pathway ERK1/2	p38, ERK1/2 [155]	1. Triplication of gene and overexpression of protein 2. Decreased expression	1. Observed in MDS and AML [163] 2. Observed in paediatric B-cell lymphoma [161,162]
DUSP1	Reduced MAPK pathways	ERK1/2, JNK and p38 [164].	Overexpression	Increased Ras-MAPK signalling in bone marrow from relapsed AML patients [180]
DUSP2	Reduced MAPK pathways	ERK1/2, JNK and p38 [164].	1. Loss of protein 2. Overexpression transcript	1. Increased ERK1/2 signalling in acture leukemia [174] 2. Observed in LGL leukemia, possible role in autoimmune disease [175]
DUSP16	Reduced MAPK pathways	ERK1/2, JNK and p38 [164].	Silencing by microRNA-24	Alters myeloid cell proliferation and differentiation [167]
PTEN	Reduced PI3K/PKB pathway	PtdIns(3,4,5)P3 at position 3 [187]	1. Homozygos and heterozygos deletion 2. Hypermethylation 3. Decreased expression 4. Increased Expression 5. Silencing by miRNA-21 or miRNA-155 6. Inactivating phosphorylation	1. Associated with poor prognosis in T-ALL [196,197] 2. Deficient PTEN expression in JMML, increased imatinyb resistance in AML [200,201] 3. Observed in AML and B-ALL [109] 4. Observed in monocytic leukemia and paediatric B-ALL [205] 5.Constitutive PKB activity in leukemia [203] 6. Correlates with poor prognosis in AML [207,208]
SHIP	Reduced PI3K/PKB pathway	PtdIns(3,4,5) P3 at position 5 [20, 187]	1. Inactivating mutation 2. Decreased expression	1. Observed in AML, leads to drug resistance [221] 2. Observed in ALL and CLL [224,225]

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Chapter 3

Hedgehog signaling maintains chemoresistance in myeloid leukemic cells

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Oncogene. 2010, 29(48): 6314-22.

ABSTRACT

The development of resistance against chemotherapy remains one of the major challenges in the clinical management of leukemia. There is still limited insight into the molecular mechanisms that maintain the chemotherapy- resistant phenotype, despite the obvious clinical relevance that such knowledge would have. In this study, we show that the chemotherapy-resistant phenotype of myeloid leukemia cells correlates with activation of the Hedgehog (Hh) pathway, whereas in chemosensitive cells, such activation is less pronounced. Importantly, the overexpression of Hh pathway components induces chemoprotection and inhibition of the pathway reverts chemoresistance of Lucena-1 cells, apparently by interfering with P-glycoprotein-dependent drug resistance. Our data thus identify the Hh pathway as an essential component of multidrug resistance (MDR) myeloid leukemia and suggest that targeting the Hh pathway might be an interesting therapeutic avenue for overcoming MDR resistance in myeloid leukemia.

INTRODUCTION

Intrinsic and acquired multidrug resistance (MDR) against chemotherapy remains a major challenge in the management of cancer in general and of leukemia in particular. Several potential molecular or cellular mechanisms responsible for MDR have been elucidated. Alterations in DNA repair, defective regulation of apoptotic gene expression, enhanced intracellular drug detoxification and overexpression of membrane drug transport proteins (for example, P-glycoprotein; P-gp) are all contributing mechanisms leading to MDR [1]. However, despite our increased understanding of MDR, current treatment options are still limited. This is at least to a certain extent because of the fact that the fundamental molecular events driving the MDR phenotype of leukemic cells remain obscure. The characterization of signaling pathways sustaining the MDR phenotype is therefore of utmost importance and such knowledge would be useful for designing rational novel therapies for MDR cancers.

The Hedgehog (Hh) signaling pathway is complex and entails two cellular receptors, that is, patched-1 (Ptch-1) and smoothened (Smo). Under unligated conditions, Ptch1 represses Smo, thereby silencing the Hh signaling pathway. Binding of the ligand (Sonic Hedgehog, Indian Hedgehog or Desert Hedgehog) to Ptch-1 alleviates Ptch-1-mediated inhibition of Smo, thereby initiating an intracellular signaling cascade leading to the activation and nuclear translocation of Gli transcription factors [2-5].

Hh signaling, originally characterized as part of the morphogenetic code, is critical for growth and differentiation during embryogenesis [2-5]. However, Hh more recently emerged as a signaling system that remains active in adulthood, in which it mediates tissue regeneration and remodeling, hematopoietic homeostasis and T-cell maturation; however, as a downside, Hh has emerged as a critical mediator in various forms of oncogenesis. Interestingly, Hh signaling seems important in normal stem cell self-renewal, as abnormal Hh expression or deficient Ptch-1 activity leads to a pre-malignant stem cell displaying unrestrained local proliferation [6, 7]. Moreover, the Hh pathway seems to be essential in the survival and expansion of Bcr- Abl+ leukemic stem cells [7]. In line with these findings, Zhao and colleagues [8] recently showed that Hh signaling has an important role in hematopoietic stem cell self-renewal and in maintenance of cancer stem cells in leukemia, although the loss of Hh signaling through conditional deletion of Smo in the adult hematopoietic compartment had no apparent effect on adult hematopoiesis in mice [9], warranting further studies as to its actual role in

the hematopoietic compartment. Together, these considerations prompt further investigation into the potential role of the Hh pathway in the pathophysiology of leukemic disease.

Various lines of evidence suggest that Hh might function in leukemia to promote hemoresistance. It has previously been shown that Hh signaling induces resistance to radiotherapy and an MDR phenotype in esophageal adenocarcinomas [10]. In apparent greement, a significant upregulation of Hh and Gli-1 expression was observed in the majority of residual solid tumors after chemoradiotherapy, suggesting that Hh signaling contributes to chemotherapy resistance in such tumors. Whether the Hh pathway has a similar role in leukemia remains lusive, and this study therefore aimed at deciphering the potential role of Hh in chemotherapy resistance in leukemia. To this end, we compared Hh pathway activity in chemotherapy-sensitive parental K562 cells and in MDR-resistant Lucena-1 daughter cells [11]. We established that Hh signaling maintains the chemoresistant phenotype in an apparently P-gpdependent manner and propose that Hh inhibition might be an attractive treatment strategy to revert (or prevent) chemoresistance in myeloid leukemia.

MATERIALS AND METHODS

Compounds, antibodies, constructs and cell lines

Vitamin D3, cyclopamine, mitoxantrone, daunorrubicin and vincristine were obtained from Sigma (St Louis, MO, USA). Gant61 was obtained from Alexis (Läufelfingen, Switzerland). Polyclonal antibodies against Gli1 and Ptch-1 were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against Smo, α-tubulin and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the monoclonal anti-P-glycoprotein antibody was obtained from Sigma. 5E1 Shh-blocking antibody was obtained from the Developmental Hybridoma Bank (Iowa City, Iowa, USA). K562, U- 937, Jurkat, KG1a, ACHN and PC3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), Lucena-1 cells were kindly donated by Professor Vivan Rumjaneck, GLC4-doxo cells were obtained from the oncology laboratory of the University of Groningen (The Netherlands). SmoM2-GFP in pMES was a generous gift from Dr Eberhart.

Cell culture and transfections

Cells were cultured in RPMI supplemented with non-essential amino acids and 10% fetal calf serum according to routine cell culture procedures. Transfections were performed using Amaxa Cell Line Nucleofector Kit V (VCA-1003) from Lonza Cologne AG (Cologne, Germany) according to the manufacturer's directions. Briefly, cells were transfected with SmoM2- GFP (5 μg DNA/1x10^6 cells) or GFP (2.5 μg DNA/1x10^6cells) expression vectors. Next, transfected cells were placed in RPMI 0.5% fetal calf serum for 16 h, and subsequently treated with vincristine for 48 h and analyzed by fluorescence-activated cell sorting or MTT assays. For reversion assays, cells were treated with mitoxantrone, doxorubicin, vincristine or imatinib, in combination with cyclopamine (10 μM), vitamin D3 (10 μM) or Gant61 (5 μM).

Cell viability assay

Cells (3x10^4) were seeded in flat-bottom 96-well plates treated with the indicated concentrations of drugs. During the last 2 h, 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) was added [21]. After incubation, the supernatant was discarded; cells were lysed in 100 μL of 0.1N HCl in

isopropanol and absorbance was measured at 570 nm in a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA).

Quantitative reverse transcription–PCR

After RNA isolation according to routine procedures, quantitative PCRs detecting the expression level of different proteins and GAPDH were performed. hP-gp-F 5'-GGCAAAGAAATAAAGCGACTGAA-3', hP-gp-R 5'-GGCTGTTGTCTCCATAGGCAAT-3', hMRP1-F 5'-CTTCTGGAGGAAT TGGTTGTATAGAAG-3', hMRP1-R 5'-GGTAGACCCAGACAAGGAT GTTAGA-3', SuFu-F 5'-CCTCCAGATCGTTGTGTCT-3', SuFu-R 5'-TCC GCATGTCAGTATCAGC-3', hGli1-F 5'-CAACTTGCCAGCTGAAGTCT-3', hGli1-R 5'-GATCCTGTATGCCTGTGGAGT-3', hGAPDH-F 5'-AAGG TGAAGGTCGGAGTCAAC-3', hGAPDH-R 5'-TGGAAGATGGTGATG GGATT-3'. Standards consisted of dilutions of RNA from K562and Lucena-1.

Nuclear extract preparation

Nuclear extracts were prepared according to routine procedures. Briefly, 2 x 10^7 cells were harvested and washed twice with ice-cold phosphate-buffered saline and resuspended in 0.2 ml of ice-cold cell extract buffer (10 mM HEPES–KOH, pH 7.9, 1.5 mM MgCl2, 10mM KC1, 0.5 mM dithiothreitol and 0.2 mM phenylmethysulfonyl fluoride). Cells were kept on ice for 10 min to allow them to swell, mixed by vortex for 10 s and centrifuged at 4°C at 14 000 g for 30 s. The supernatant was discarded, and the pellet was resuspended in 30 ml of nuclear extraction buffer (20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethysulfonyl fluoride), placed on ice for 20 min and centrifuged at 4°C at 14 000 g for 2 min. The remaining supernatant was used as nuclear extract in western blotting assays.

Western blotting analysis

Cells (2.5x10^7) were lysed in 200 µl of lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Tween-20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM O-Vanadate, 1 mM NaF and protease inhibitors (1 mg/ml aprotinin, 10 mg/ml leupeptin and 1mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride- hydrochloride) on ice for 2 h. Protein extracts were cleared by centrifugation and the protein concentration was determined using the Lowry

method [22]. An equal volume of 2xSDS gel loading buffer (100mM Tris–HCl (pH 6.8), 200mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added and samples were boiled for 10 min. Cell extracts were resolved by SDS–PAGE (12%) and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in TBS/0.05% Tween-20 and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilutions. After washing in TBS/0.05% Tween-20, membranes were incubated with antirabbit, antigoat and antimouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions in blocking buffer for 1 h. Blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS-3000 imaging system.

Statistical analysis

Unless otherwise indicated, all experiments were conducted in triplicate and the results shown in the graphs represent the mean and s.e. Data were analyzed by ANOVA. Western blots represent three independent experiments.

RESULTS

Sensitivity to chemotherapeutic drugs of K562 and Lucena-1 cells

Studying therapy-induced drug resistance in vitro remains a challenge, but Lucena-1 cells have previously been described as vincristine-resistant leukemia cells derived from vincristine- sensitive K562 parental cells [11] (Figure 1); indeed, in our hands, the microtubule polymerization inhibitor vincristine, as well as other therapeutically important drugs such as the DNA intercalating mitoxantrone and doxorubicin, efficiently kills K562 cells, whereas these drugs only minimally affect Lucena-1 survival (Figure 1a). Chemoresistance of Lucena-1 was accompanied by upregulation of drug transporters P-gp and MRP-1, (Figure 1b) whereas the P-gp inhibitor verapamil partially reverted drug resistance. We concluded that the comparison of K562 and Lucena-1 constitutes a valid model to study induced resistance for classical chemotherapy.

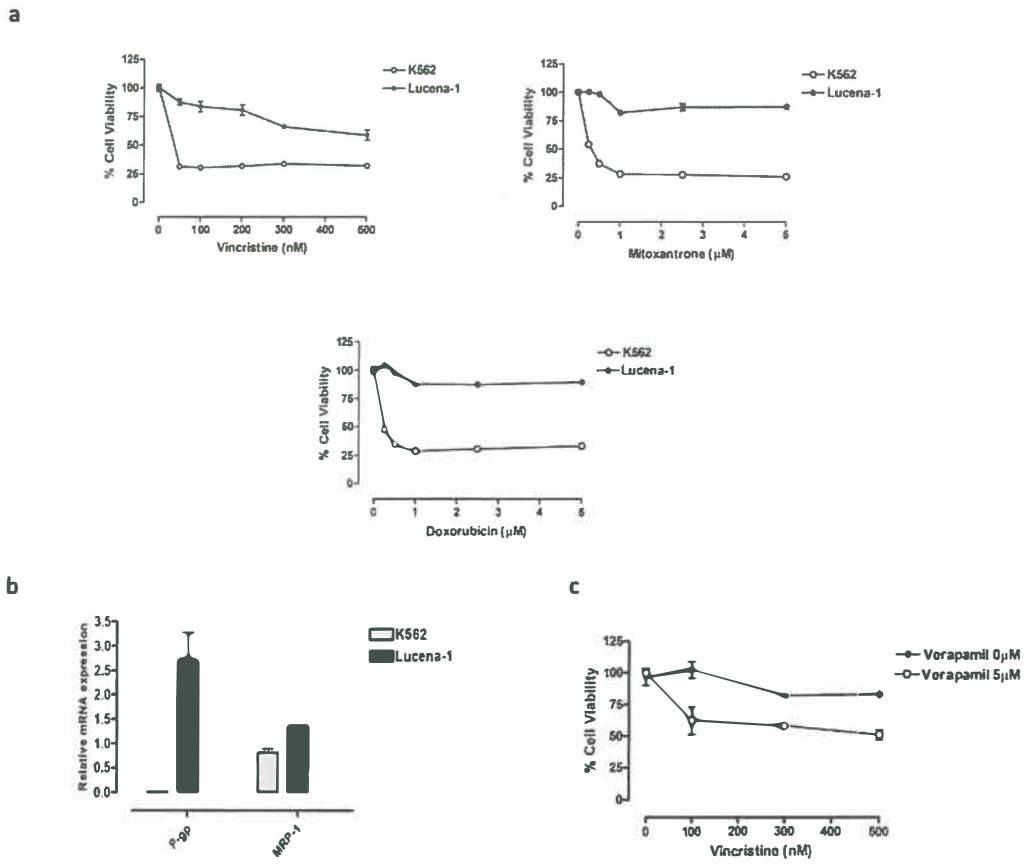

Figure 1. Cell viability of K562 and its multidrug-resistant counterpart Lucena-1 cells in the absence and presence of hemotherapeutic drugs. (a) K562 and Lucena-1 cells were treated with vincristine (0–500 nM), mitoxantrone (0–5 μM) and doxorubicin 0–5 μM) for 48 h, and cell viability was assessed by MTT reduction assays. (b) Expression level of efflux pumps (P-gp and MRP-1) in 562 and Lucena-1 cells as determined by quantitative PCR. (c) Lucena-1 cells were treated with vincristine in the absence or presence f the pump inhibitor verapamil (5 μM) and cell viability was assessed by MTT reduction assays. Shown are mean ± s.e.m. (n≥3).

Different sensitivity to chemotherapeutic drugs is determined by the Hh pathway

We decided to study whether activation of Hh signaling is a characteristic of chemoresistance under our experimental conditions. Importantly, as shown in Figure 2a, the *bona fide* Hh target gene Gli1 displays sevenfold increased mRNA levels in Lucena-1 cells, compared with the chemosensitive parental line, whereas Sufu expression levels (which are

suggested to be inversely associated with Hh signaling [12], although this is not generally established) were reduced by twofold. At the protein level, we observed that Sonic Hedgehog was induced, together with the classical Hh target genes Gli and Ptch-1, in chemoresistant cells (Figure 2b). Finally, nuclear extracts showed the hallmark appearance of full-length Gli1 in the nuclei of chemoresistant cells, but not in those of chemosensitive cells (Figure 2c). Overall, these data show that the chemotherapy-resistant phenotype is accompanied by a constitutively activated Hh signaling pathway.

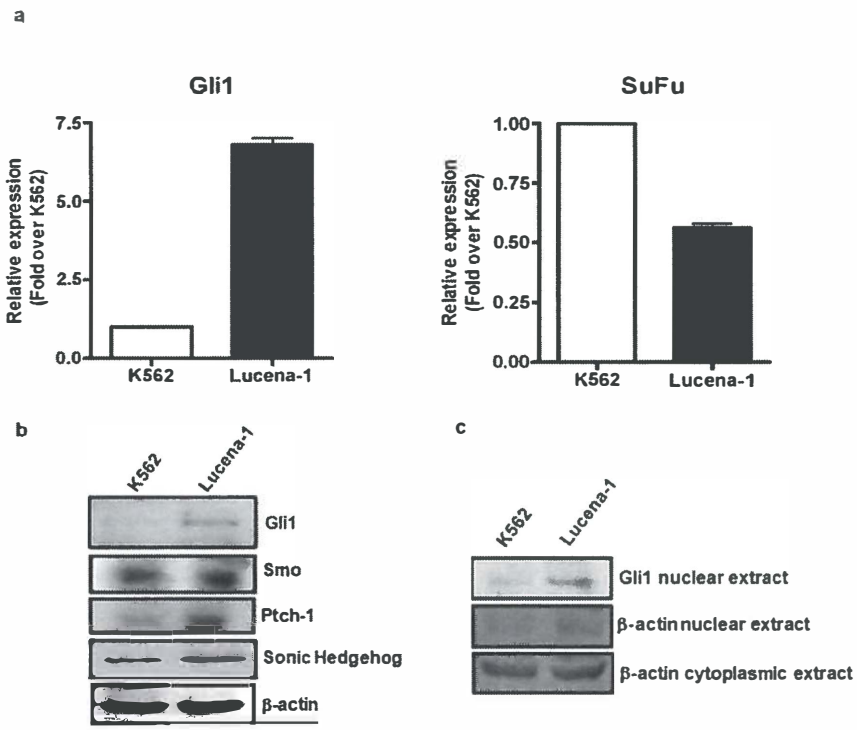

Figure 2. Hedhehog (Hh) signaling in K562 and its multidrug-resistant counterpart, Lucena-1. (a) K562 and Lucena-1 present differences in Hh pathway components. Shown are Gli1 and SuFu mRNA expression levels as determined by quantitative PCR. (b) Expression levels of the Hh pathway components Gli1, Ptch-1, Smo and Shh on the protein level as determined by western blot analyses. (c) Gli1 protein levels in nuclear extracts of K562 and Lucena-1 as determined by western blotting.

The Hh pathway maintains the multidrug-resistant phenotype

To investigate the importance of Hh signaling in chemoresistance, K562 cells were transfected with SmoM2-GFP (a constitutively active Smo

variant) or GFP-overexpressing constructs. The transfections were effective (efficiency of approximately 40% (data not shown)) and resulted in increased Gli1 and Ptch-1 expression levels (Figure 3a). As shown in Figure 3b, induction of Hh signaling by SmoM2 overexpression protected K562 leukemia cells from vincristine treatment. Furthermore, vincristine treatment increased the percentage of SmoM2-GFP-positive cells, but not that of GFP-only transfected cells (Figure 3c), suggesting that Hh pathway activity might be an important component in the acquisition of resistance to classical chemotherapy in leukemia.

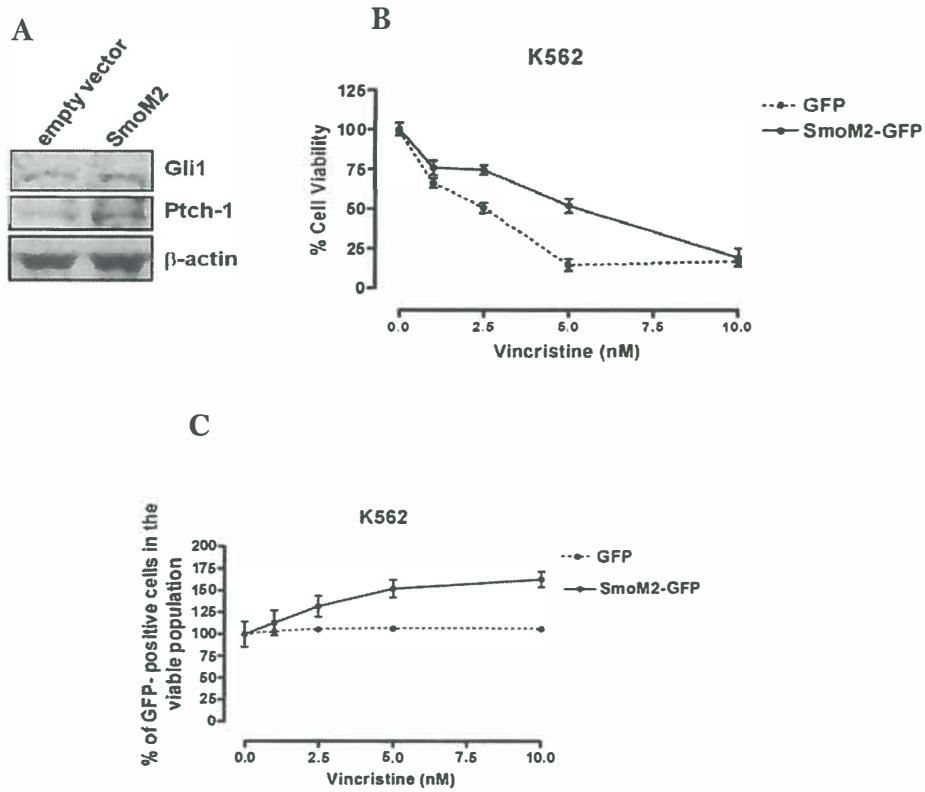

Figure 3. Overexpression of Hh pathway components is sufficient for chemoprotection of K562 cells. (a) K562 cells were transfected with SmoM2-GFP or GFP expression constructs, after which Gli1 and Ptch-1 levels were determined by western blotting. (b) K562 cells transfected with SmoM2 are protected against vincristine-induced cell death. (c) Vincristine treatment increased the percentage of SmoM2-GFP-positive cells but not that of GFP-only transfected cells. Shown are mean±s.e.m. (n=3).

Pharmacological inhibition of Hh signaling reverts resistance in Bcr-Abl$^+$ cells

We observed that chemoresistance was accompanied by induction of Hh signaling and that activation of Hh signaling is sufficient for acquiring chemoresistance; however, therapeutically, it is more interesting to investigate to which extent the chemoresistant phenotype actually depends on activation of this pathway. Thus, we pharmacologically inhibited the Hh pathway in Lucena-1 cells and analyzed the effect on chemoresistance. As shown in Figure 4a, Hh pathway inhibitors cyclopamine [13] and vitamin D3 [3] indeed inhibited Hh pathway activity in Lucena-1 cells (using Ptch-1 levels as read out). Importantly, both Hh inhibitors did not affect cell survival *per se* in concentrations up to 10 μM (Figure 4b). The presence of Hh inhibitors, however, substantially enhanced the sensitivity of Lucena-1 cells to vincristine to levels not markedly different from those of the parental line (IC50 approximately 10 nM in both cases; compare Figures 1 and 4c). This effect was not restricted to microtubules system-targeted chemotherapy, as doxorubicin- or mitoxantrone sensitivity was also restored to the levels of the parental cell line following inhibition of the Hh pathway.

To provide further proof for the notion that inhibition of the Hh pathway may revert chemoresistance, Lucena-1 cells were treated with vincristine in combination with Gant61 (inhibitor of Gli1 transcriptional activity) [14]. As shown in Figure 4d, Gant61 efficiently inhibited Hh pathway activity and the inhibition of Gli1 transcriptional activity by Gant61 indeed reverses chemoresistance, suggesting that the increased level of Gli1 (Figure 2a) is important to maintain the resistant phenotype in Lucena-1 cells. To determine whether the effect of Hh inhibition is specific for MDR leukemia cells, the combinations mentioned above (Hh pathway inhibitors plus vincristine) were also tested in K562 cells. As shown in Figure 4e, Hh inhibitors hardly effected vincristine-induced cell death (at least in comparison with the effect observed in Lucena-1 cells (Figure 4c), suggesting that the chemosensitization induced by Hh pathway inhibitors is specific for MDR cells. Overall, it seems that pharmacological inhibition of the Hh pathway might constitute an interesting opportunity for chemosensitization in the management of MDR myeloid leukemic disease.

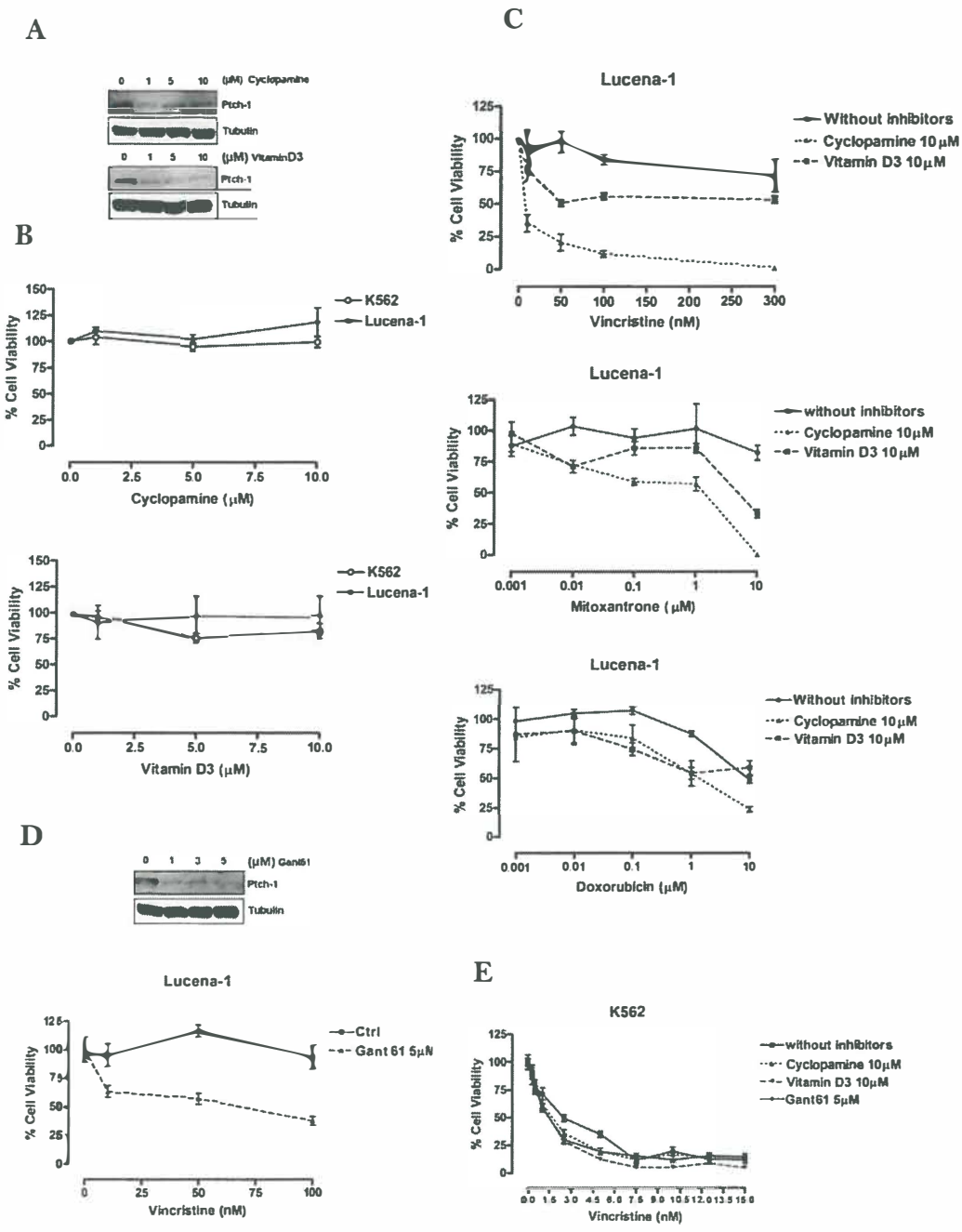

Figure 4. Hh signaling inhibitors counteract chemoresistance of Lucena-1 cells. (a) Ptch-1 expression with and without cyclopamine or vitamin D3 treatment. (b) Cyclopamine and/or vitamin D3 treatment (0–10 μM for 48 h) does not affect the viability of K562 and Lucena-1 cells as assessed by MTT reduction assays. (c) Both cyclopamine and vitamin D3 sensitize Lucena-1 cells for chemotherapeutic drugs.

Lucena-1 cells were treated with increasing concentrations of vincristine, mitoxantrone or Doxorubicin in the presence of 10 μM cyclopamine or vitamin D3 for 48 h. (d) Treatment of Lucena-1 cells with the Gli-1 inhibitor Gant61 inhibits Ptch-1 levels and reverts chemoresistance to vincristine. (e) Hh pathway inhibitors do not sensitize K562 cells for vincristine. K562 cells were treated with increasing concentrations of vincristine, in the presence of cyclopamine, vitamin D3 or Gant61 for 48 h. Shown are mean ± s.e.m. ($n \geq 3$).

Myeloid specificity of chemosensitization by inhibition of Hh signaling

To address the specificity of the effects observed, a more or less random panel of leukemia cells and solid cancers was exposed to pharmacological Hh inhibition, and effects of this treatment on chemosensitivity were assessed. As evident from Figure 5, neither solid cancers nor T-cell leukemia showed much evidence for such chemosensitization. Although these results show that alleviation of therapy resistance using Hh inhibitors is restricted to the myeloid compartment, they also provide good evidence that effects observed are specific and cannot be explained from an off-action of the pharmacological inhibitors involved. Pharmacological inhibition of Hh signaling interferes with P-gp expression Subsequently, we were interested as to whether we could dissect the point of interaction of Hh signaling with the classical cellular physiology of leukemic cell. As Hh appears to affect the resistance of MDR leukemia cells, we hypothesized that targeting the Hh pathway might reduce P-gp levels. As shown in Figure 6, all Hh inhibitors indeed reduced expression levels of P-gp, although to different extents.

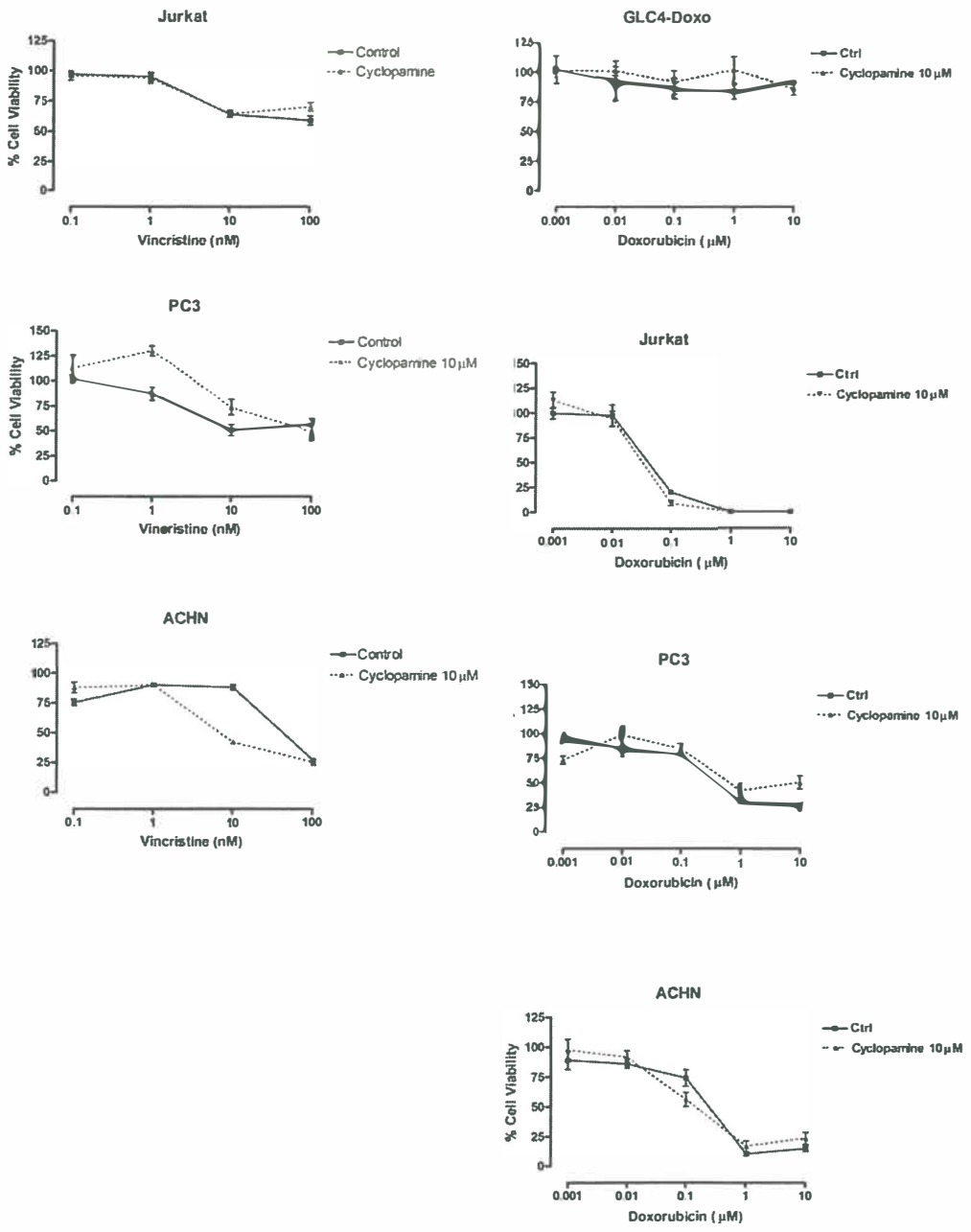

Figure 5. Cyclopamine -induced sensitization is not a general mechanism in cancer cells. Jurkat, ACHN, PC3 and GLC4-Doxo cells were treated with cyclopamine (10 µM) in combination with different concentrations of vincristine or doxorubicin, and cell viability was evaluated by MTT reduction assays. Shown is the mean ± s.e.m. (n=3).

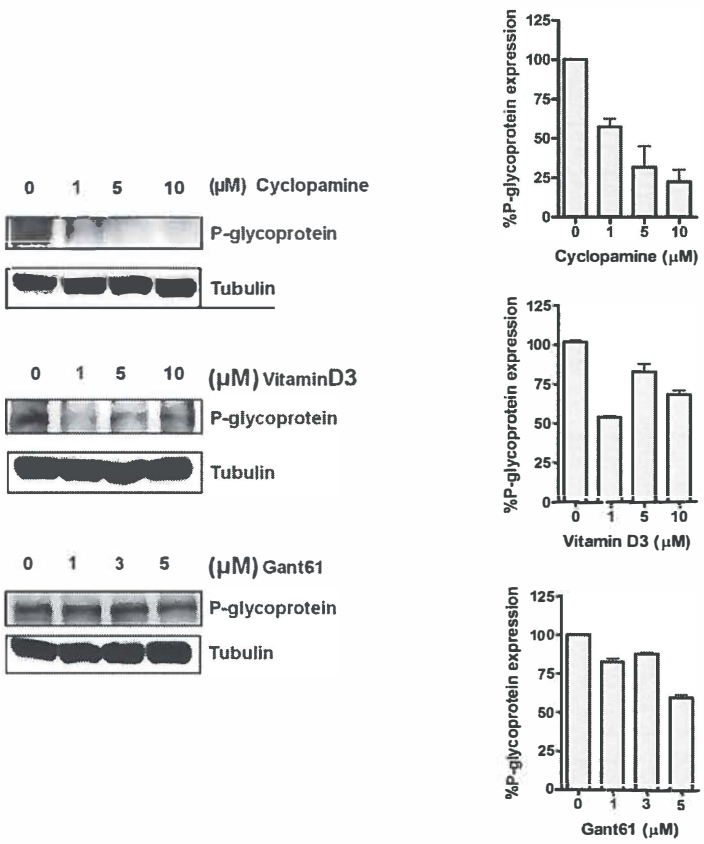

Figure 6. Hh pathway inhibitors sensitize Lucena-1 cells dependent on P-glycoprotein expression. P-glycoprotein expression in Lucena-1 cells was analyzed after 24 h of treatment with cyclopamine, vitamin D3 and Gant61. Shown on the left is a representative experiment, whereas the quantification of three independent experiments is shown on the right (mean ± s.e.m.).

DISCUSSION

Intrinsic and acquired MDR against chemotherapy remains one of the major problems in the management of leukemic disease [10, 15-18]. The majority of leukemia patients (75%) of <60 years do present an initial complete remission after chemotherapy. However, 60% of these responders will ultimately present disease relapse, usually occurring within the first 2 years after the start of treatment [18]. Despite our increased understanding of MDR and the elucidation of several underlying mechanisms [1, 6, 19], treatment options are still limited. The characterization of signaling pathways sustaining MDR is thus essential for designing rational novel therapies for MDR leukemia. In this study, we show that the chemotherapy-resistant phenotype of myeloid leukemia cells correlates with the activation of Hh signaling, that overexpression of Hh pathway components induces chemoprotection and that inhibition of the pathway reverts chemoresistance of Lucena-1 cells. Our data thus identify the Hh pathway as an essential component of MDR leukemia, and suggest that targeting the Hh pathway might be an interesting therapeutic avenue for overcoming MDR resistance in myeloid leukemia. These data provide a mechanistic explanation as to the previous observation that inhibition of the Hh pathway in Kasumi1, -3 and TF1 myeloid cell lines increased sensitivity to suboptimal doses of the antimetabolite cytarabine [20]. We propose that activation of the Hh pathway is both sufficient and essential for resistance to classical chemotherapy in myeloid leukemia.

The mechanism of action of Hh inhibitors is probably dependent on its effect on P-gp expression levels (as shown in Figure 6). This immediately explains that the chemosensitization induced by Hh inhibitors in combination with vincristine is only observed in Lucena- 1 cells and is not (very) effective in K562 or GLC4-doxo cells. These latter cells express MRP-1, but not P-gp, and sensitization can consequently not be induced in these cells by Hh inhibitors that target P-gp. Overall, these data indicate an important role of the Hh pathway in the maintenance of the MDR phenotype in myeloid leukemias. Our findings might be relevant for the treatment of MDR leukemia, but in addition, our data may be relevant for the early detection of resistance. Indeed, the expression of sentinel markers (for example, Ptch-1) of activation of the Hh pathway in peripheral blood myeloid leukemia cells may be an indication that a patient is developing chemoresistance and is possibly a useful predictor of treatment failure, necessitating the switch to alternative therapy.

Further studies are obviously essential to address the validity of these latter points.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

KCS Queiroz is supported by the Maag-, Lever-, Darmstichting. CV Ferreira is supported by a research fellowship from CNPq. MP Peppelenbosch and CA Spek acknowledge the support of the Top-Institute pharma. We are grateful to Professor Vivian Rumjanek (Federal University of Rio de Janeiro) for donating Lucena-1 cells.

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Chapter 4

Phosphoprotein levels, MAPK activities and NFκB expression are affected by fisetin

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J Enzyme Inhib Med Chem. 2007, 22(4): 439-44.

ABSTRACT

Flavonoids, polyphenolic phytochemicals, are ubiquitous in plants and are commonly present in the human diet. They may exert diverse beneficial effects, including antioxidant and anticarcinogenic activities. The present study was designed to evaluate three biomolecules that play important roles in the apoptotic process: mitogen-activated protein kinases, protein phosphatases and NFκB, using HL60 cells treated with fisetin as an experimental model. Our results demonstrated that cells treated with fisetin presented high expression of NFκB, activation of MAPK p38 and an increase of phosphoprotein levels; inhibition of enzymes involved in redox status maintenance were also observed. Our findings reinforce the hypothesis that fisetin is likely to exert beneficial and/or toxic actions on cells not through its potential as antioxidant but rather through its modulation of protein kinase and phosphatase signaling cascades. Additionally, our results also indicate that the cellular effects of fisetin will ultimately depend on the cell type and on the extent to which they associate with the cells, either by interactions at the membrane or by uptake into the cytosol.

INTRODUCTION

Flavonoids are polyphenolic compounds widely found in plants [1]. Components of fruits, vegetables and beverages, such as wine and tea, many flavonoids are present in a regular diet [2]. Flavonoids exhibit a variety of effects such as inhibition of malignant cell growth [1], regulation of lymphocyte activation, cell proliferation and differentiation [2-4]. These biological effects of flavonoids on cells can be due to the inhibition of different key enzymes. For these reasons, the flavonoids can be considered potential compounds in the selective blocking of signal transduction pathways and in the design of more potent analogues for use in proliferative disease therapies.

Several studies have demonstrated that, depending on their structures, flavonoids can be potent inhibitors of several kinases involved in signal transduction, mainly protein kinase C (PKC) [5] and tyrosine kinases [6]. On the other hand, some flavonoids can activate cell differentiation through activation of the Ras-ERK cascade [7].

Fisetin is a common dietary component found in several fruits and vegetables [8]. Some authors have demonstrated different biological activities for this flavonoid: inhibition of topoisomerase II, an essential nuclear enzyme for DNA replication [9, 10], neuroprotective, cardioprotective and anti-carcinogenic activities, which have been attributed to its antioxidant properties [7, 11-13], inhibition of cellular proliferation and *in vitro* angiogenesis [14], induction of apoptosis in leukemic cells [13]. Recently, Haddad et al. [15] have demonstrated that fisetin caused cell cycle arrest (G2/M) in a prostate cancer human cell line (PC3). In addition, fisetin inhibited glucose uptake in a competitive manner in a myeloid cell (U937), which indicated that this flavonoid could be used as an alternative blocker of glucose uptake *in vitro* [16]. The present study was designed to evaluate three biomolecules that play important roles in the apoptotic process: mitogen-activated protein kinases (MAPKs), protein phosphatases and NFκB, using HL60 cells treated with fisetin as an experimental model. Our results demonstrated that cells treated with fisetin presented high expression of NFκB, activation of MAPK p38 and an increase of phosphoprotein levels; inhibition of enzymes involved in redox status maintenance was also observed.

MATERIALS AND METHODS

Materials

HL60 cells were from ATCC (Rockville, MD) and fisetin (Figure 1) was from Sigma Chemical Co. (St Louis, MO). The polyclonal antibodies against antiphosphop38 mitogen-activated protein kinase (p38), antiphospho-p42/44 (ERK 1/2), antiphospho-c-jun NH2- terminal protein kinase (JNK), antiphospho-MAPK/ ERK kinase 1 (MEK1), antirabbit and antimouse peroxidase conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture

HL60 cells were routinely grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (10,000 U/mL penicillin and 10mg/mL streptomycin) and 1% glutamine, grown at 37°C under a humidified 5% CO_2 atmosphere. In all experiments 3 x 10^5 cell/mL were seeded, and after 72 h the cells were treated with fisetin for 24 h. Fisetin dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium and adjusted to a final DMSO concentration of 0.1%.

Cell viability

Cell viability was assessed by the trypan blue dye exclusion and the MTT reduction assays as previously described [17].

Western blotting

Cells (3 x 10^7) were lysed in 200 µL cell lysis buffer (50mM Tris-HCl pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1mM o-vanadate, 1mM sodiumfluoride, and protease inhibitors (1mg/mL aprotinin, 10mg/mL leupeptin, and 1mM phenylmethylsulfonyl fluoride (PMSF)) for 2 h on ice. Protein extracts were cleared by centrifugation, and the protein concentration was determined using the Lowry method [18]. Twice the volume of sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to the samples and the mixture boiled for 10 min. Cell extracts, corresponding to 3 x 10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and

transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS) -Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with antirabbit or antimouse horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence (ECL).

Antioxidant enzyme activities

Total superoxide dismutase (SOD) activity was determined from the rate of inhibition of ferrocytochrome c oxidation, at 550 nm, in a standard reaction medium [19]. The manganese superoxide dismutase (MnSOD) activity was measured after inhibition of the Cu/Zn isoenzyme by addition of 1mM KCN [20]. Catalase activity was determined by measuring the decrease in absorption of H_2O_2 at 240 nm [21]. Glutathione peroxidase (GPX) activity was determined by measuring the NADPH oxidation rate in the presence of GSH and GSH reductase [22]. Marker enzyme activties for oxidative stress Aconitase activity was measured at 25°C by following the change in the absorption at 340 nm, due to $NADP^+$ reduction [23]. Fumarase activity was measured at 25°C by following the increase in absorbance at 240 nm at 25°C in a standard reaction mixture [24]. All the measurements were carried out in a UV-VIS spectrophotometer (Hitachi, model U-2001).

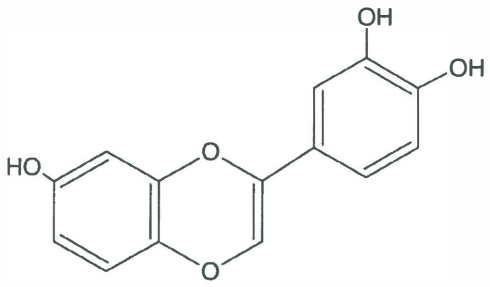

Figure 1. Molecular structure of fisetin.

Statistical evaluation

The Western blots represent three independent experiments. Cell viability was expressed as the mean ± standard error of three independent experiments run in triplicate. Data for each assay were statistically evaluated by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Differential effect of fisetin on HL60 cells and normal human lymphocyte viabilities

We have previously described cytotoxic effects and mechanism of action of different compounds on cancer cells [25-27]. Other natural products have also been reported as important sources of potential chemotherapeutic agents [28-30]. Flavonoids, widely distributed in vegetables, fruits, and wine, have been shown to exert anticarcinogenic effects [10, 13, 15, 31]. However, the molecular mechanisms by which flavonoids can act against cancer cells need to be elucidated. To establish the specificity of fisetin action on HL60 cells we checked, in parallel, the effect of this compound on normal human lymphocytes viability using the MTT assay. It was observed that after 24 hours of fisetin-treated HL60 cells, the mitochondrial activity was decreased, displaying an IC50 value around 30 μM (Figure 2). In agreement with other authors [13], it was also observed that fisetin induced HL60 cells death by apoptosis. Interestingly, human lymphocyte viability remained unchanged, even in the presence of fisetin at concentrations up to 200 μM. These results suggest that fisetin can be an interesting candidate for cancer treatment with a cellular-specific mechanism of action.

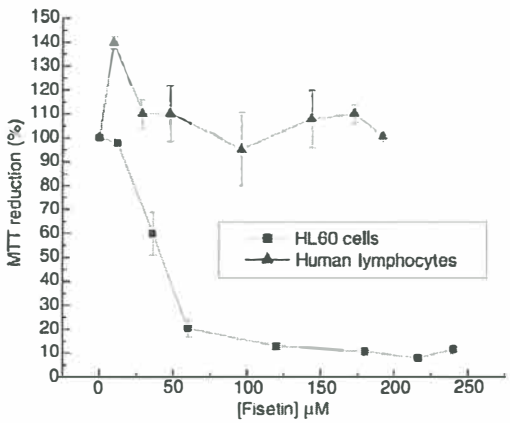

Figure 2. Cytotoxicity of fisetin in leukemic cells and normal human lymphocytes. HL60 cells (■) and normal human lymphocytes (▲) were treated with different concentrations of fisetin for 24 h. In the absence of fisetin, the MTT reduction was considered as 100%. The experiment was performed in a 24-well plate. Results represent the means ± standard error of three experiments run in triplicate ($p < 0.05$).

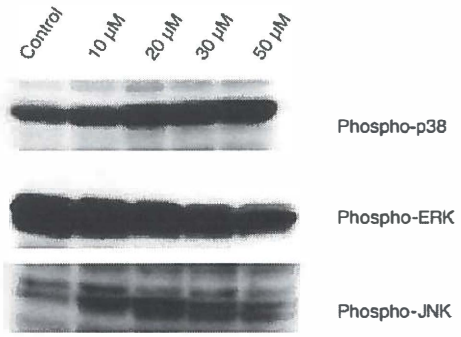

Figure 3. Effect of fisetin on MAPKs phosphorylation in HL60 cells. Cells were treated with different concentrations of fisetin (10, 20, 30 and 50 μM). Soluble lysates were matched for protein content and analyzed by Western blot. One representative immunoblot of three independent experiments is presented.

Effect of fisetin on MAPKs phosphorylation and NFκB expression in HL60 cells

To obtain more insight into the molecular mechanisms mediated by fisetin on HL60 cells, we examined the phosphorylation state of total proteins and MAPKs, in response to fisetin at concentrations up to 50 μM. Fisetin caused activation of p38 and JNK MAPKs, while ERK was inhibited (Figure 3). Williams et al. [32] have demonstrated that flavonoids and their metabolites differentially acted on PI3-kinase, Akt/ protein kinase B (Akt/PKB), tyrosine kinases, PKC, and MAPK signalling cascades. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression.

In addition, we also observed that cells treated with fisetin presented high expression of NFκB (Figure 4). Decrease in MAPKs phosphorylation (Figure 3) and in the expression of NFκB p65 (Figure 4) in HL60 cells at fisetin concentrations higher than 20 μM could be ascribed to different steps of apoptosis. Our results indicate that depending on the fisetin concentration two steps of apoptosis can be reached: early and late apoptosis. Apparently, fisetin concentrations up to 20 μM caused early apoptosis, that was reinforced by the overexpression of NFκB. Recently, Kanno et al. [33] demonstrated that the overexpression of NFκB is a pivotal event for apoptosis in HL60 cells induced by the flavonoid naringenin. It has been shown that the transcription factor NFκB participated in cell growth, differentiation and inflammatory responses

induced by different signals related to the regulation of apoptosis and neoplastic transformation [34, 35]. The pro- and antiapoptotic regulatory functions of NFκB have been shown to depend on the cell type, the differentiation state of the cell, and the nature of the apoptotic stimulus [35]. Our data provided evidence that the overexpression of the subunit NFκB p65 in cell death was associated with ROS generation. Some authors observed that ROS per se were potent inducers of apoptosis [36] and that the hydrogen peroxideinduced apoptosis required the release of mitochondria- derived ROS and the activation of NFκB [37]. Our results demonstrating the ability of NFκB p65 overexpression to induce apoptosis are in agreement with published data implicating NFκB to the induction of cell death in certain cells such as neurons, Schwann cells, prostate carcinoma, and embryonic kidney cells [38-42].

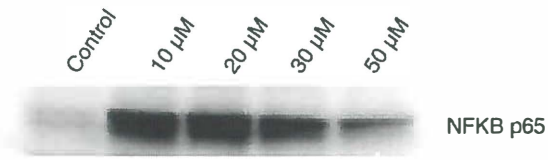

Figure 4. Fisetin changes the expression of NFκB p65. After treatment of HL60 cells with different concentrations of fisetin for 24 h, equal amounts of protein (50μg) of total lysates were subjected to immunoblot analysis with NFκB (p65) antibodies.

Effects of fisetin on protein phosphorylation in HL60 cells

In order to analyze the phosphorylation state in HL60 cells treated with fisetin, we examined the tyrosine and threonine phosphorylation on the cellular proteins. Phosphorylation of both residues increased in the cells treated with fisetin, except for a decrease in tyrosine phosphorylation at 50 mM fisetin (Figure 5). Our results indicated that the fisetin action in HL60 cells was accompanied by an increase in tyrosine and threonine phosphorylations. We have observed that fisetin inhibited cytosolic phosphatase activities in HL60 cells (not shown).

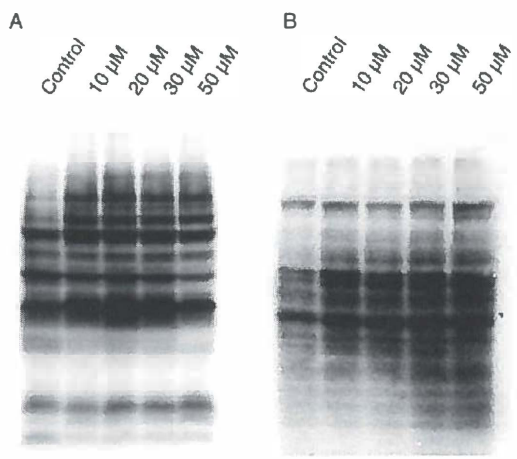

Figure 5. Effect of fisetin on protein phosphorylation levels. Cells were treated with fisetin (10, 20, 30 and 50 μM), and the tyrosine (A) and threonine (B) phosphorylations were evaluated by immunoblotting. One representative immunoblot of three independent e xperiments is presented.

Fisetin induces oxidative stress and decrease in antioxidant enzymes activities in HL60 cells

In order to analyze the cellular redox status after treatment of HL60 cells with fisetin we quantified the activities of aconitase, fumarase, catalase, glutathione peroxidase and two isoforms of superoxide dismutase (SOD), i.e. the MnSOD (mitochondrial isoform), and the CuZnSOD (cytosolic isoform). Treatment of HL60 cells with fisetin resulted in inactivation of mitochondrial aconitase, an enzyme sensitive to oxidative stress, but not fumarase, a mitochondrial enzyme sensitive to oxidative stress (Table I). Fisetin caused also an expressive decrease in the antioxidant enzymes catalase, MnSOD, CuZnSOD and GPX.

Our results suggest that fisetin can induce oxidative stress through ROS production. ROS can lead to cell death through inactivation of mitochondrial aconitase, an iron-sulfur (Fe-S) protein [43]. Recent studies showed that ROS are emerging as obligatory mediators of cell death signaling in response to stimulation of TNF receptors and induction of JNK and p38 signaling [44-47]. A MAPK phosphatase (MKP) was identified as a critical molecular target of ROS during TNFa-induced apoptosis, due to oxidation of an essential cysteine residue to sulfenic acid [46]. MKP plays a critical role in the regulation of the activity of MAPKs [48, 49]. Thus, ROS-dependent inhibition of MKPs caused persistent activation of JNK by TNFα, and,

ultimately, programmed cell death via either a necrotic or an apoptotic pathway [47]. These findings are in agreement with our results since, besides activating JNK and p38, fisetin also caused an increase of phosphoprotein levels which can be due to either inactivation of protein phosphatases or activation of protein kinases, activities which are highly sensitive to oxidant agents.

Table I. Effects of fisetin on antioxidant enzymes activities of HL60 cells. Cells were treated with fisetin (100 μM) and enzyme activities were determined as described in Materials and Methods.

	Specific Acitivies	
Enzymes	Control	+ Fisetin
Aconitase	2.32 mU/mg	0.11 mU/mg
Fumarase	0.029 mU/mg	0.029 mU/mg
Catalase	0.23 mU/mg	0.11 mU/mg
GPX	0.66 mU/mg	0.22 mU/mg
MnSOD	6.22 mU/mg	2.46 mU/mg
CuZnSOD	11.74 mU/mg	3.33 mU/mg

CONCLUSION

In summary, our results have reinforced the hypothesis that fisetin was likely to exert beneficial and/or toxic actions on cells not through its potential to act as antioxidant but rather through its modulation of protein kinase and phosphatase signaling cascades. Additionally, our results also indicated that the cellular effects of fisetin ultimately depended on the cell type, and on the extent to which it associated with the cells, either by interactions with the membrane or by uptake into the cytosol.

ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - Proc. 04/15041-5, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), CAPES postgraduate scholarship to RRRS, and FAPESP postgraduate scholarships to KCSQ and ACSS.

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Chapter 5

Cytotoxicity of apigenin on leukemia cell lines: implications for prevention and therapy

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Cell Death and Disease. 2010, 1, e19.

ABSTRACT

Natural food-based compounds show substantial promise for prevention and biotherapy of cancers including leukemia. In general their mechanism of action remains unclear, hampering rational use of these compounds. Herein we show that the common dietary flavonoid apigenin has anticancer activity, but also may decrease chemotherapy sensitivity, depending on the cell type. We analyzed the molecular consequences of apigenin treatment in two different types of leukemia, the myeloid and erythroid subtypes. Apigenin blocked proliferation in both lineages through cell cycle arrest in G2-M phase for myeloid HL60 and G0/G1 phase for erythroid TF1 cells. In both cell lines the JAK/STAT pathway was one of major targets of apigenin. Apigenin inhibited PI3K/PKB pathway in HL60 and induced caspase-dependent apoptosis. In contrast, no apoptosis was detected in TF1 cells, but initiation of autophagy was observed. The block in cell cycle and induction of autophagy observed in this erythroleukemia cell line resulted in a reduced susceptibility towards the commonly used therapeutic agent vincristine. Thus, the present study shows that although apigenin is a potential chemopreventive agent due to the induction of leukemia cell cycle arrest, caution in dietary intake of apigenin should be taken during disease as it potentially interferes with cancer treatment.

INTRODUCTION

Apigenin (4',5,7-trihydroxyflavone), a common dietary flavonoid abundantly present in fruits and vegetables, displays promising biological effects such as prevention and therapy of prostate cancer, suppression of tumorigenesis and angiogenesis in melanoma [1] and breast, skin and colon carcinomas [2]. It has been shown to exhibit antitumoral effects in leukemia cells via induction of apoptosis through activation of caspases, inhibition of fatty acid synthase and topoisomerase and modulation of Bax and Bcl-2 expression [3] and indeed a variety of studies have documented anti-leukemic effects of apigenin [4-7]. Despite apigenin seems to be promise as a chemopreventive in leukemia, a rational design of trails investigating its potential in this respect awaits further elucidation of its molecular mode of action.

Studies in breast cancer cells have identified some of the potential key protein kinases potentially responsible for apigenin effects, in particular PI3K, PKB and ERK1/2 and other upstream kinases involved in cancer development and progression [8]. However, the effects of apigenin on these kinases in leukemia have not been investigated.

The promise of apigenin now urgently calls for investigations as to its molecular mode of action in leukemia cellular physiology. This consideration prompted us to analyze the molecular pathways activated by apigenin treatment on the leukemic cell. To this end we established the induction of cell cycle arrest and cell death in two models of leukemic disease, myeloid (HL60 cells) and erythroid (TF1 cells).

Abbreviations: AIF, apoptosis-inducing factor; Atg, autophagy-related (Atg) genes Bax, Bcl-2 associated x protein; Bcl-2, B-cell lymphoma protein 2; c-myc, transcriptional regulator protein; Cdc2, cell division control protein 2; CDKs, cyclin-dependent kinases; ERK1/2, extracellular signal-regulated protein kinase 1/2; GSK-3β, glycogen synthase kinase-3 β; JAK2, janus family of tyrosine kinase 2; LC3B, Light Chain 3 isoform B; LMWPTP, low molecular weight protein tyrosine phosphatase; LY294002, PI3K inhibitor; MAPKp38, mitogen-activated protein kinase p38; mTOR, mammalian target of rapamycin, a Ser/Thr protein kinase; p21, tumor suppressor protein; p70S6K, 70-kDa ribosomal protein S6 kinase; PARP, poly (ADP-ribose) polymerase; PDK-1, phosphoinositide- dependent protein kinase 1; PI3K, Phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homolog; S6, S6 ribosomal protein; SHP-2, Src homology 2 domain-containing phosphotyrosine phosphatase 2; Src, Src family of protein tyrosine kinases; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; ZVADfmk, pan caspase inhibitor.

MATERIAL AND METHODS

Cell lines and reagents

HL60, K562 and TF1 cells were purchased from ATCC (Rockville, MD, USA). Apigenin was from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies against AIF, p-PKB(Ser-473), p-cdc2(Thr15), CDK4, CDK6, cleaved caspase 3, cleaved PARP, caspase 8, caspase 7,c-myc, p-GSK-3β(Ser-9), p-PI3K p85, p-PDK(ser241), p-JAK2(Tyr1007/1008), p-Src(Tyr416), p-STAT3(Tyr705), p-STAT5(Tyr-694) p-p38(Tyr108/182), p-SHP2(Tyr542), p-PTEN(Ser380), p-mTOR(Ser2448), p-p70S6K(Thr389), p-S6(Ser235/236), Beclin-1, LC3BI/II, Atg5, Atg7, Atg12, antirabbit and antimouse peroxidase-conjugated antibodies were from Cell Signaling Technology (Beverly, MA, USA). p21, TNFR1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against LMWPTP was purchased from Abcam (Cambridge, MA, USA). Apigenin and vincristine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase inhibitor Z-VADfmk and apoptosis kit detection (annexin V-FITC and Propidium iodide) were from BD Biosciences (San Diego, CA, USA). The PI3K inhibitor LY294002 was from Alexis (Läufelingen, Switzerland) and TNFα was from Biovision Inc. (Mountain View, CA, USA).

Cell culture

Leukemia cells were cultured in RPMI 1640 containing 100U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum, at 37°C in a 5% CO_2 humidified atmosphere. For TF1 cells, 5 ng/mL GMCSF was added to medium.

Human lymphocytes were obtained from healthy volunteers and isolated by density through Ficoll Paque® gradient. Mononuclear cells were harvested and cultured in RPMI1640 containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum and concanavalin A (5 μg/mL) for 48 h when the treatment was done.

MTT assay

Cell viability was assessed by MTT reduction assay as described by Mosmann (1983) [34]. Non-treated cells were taken as 100% of viability and IC50 values were determined from three independent experiments.

Western blotting

Cells were lysed in cell lysis buffer [50mM Tris-HCl pH 7.4 containing 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1 mM EGTA, 1 mM o-vanadate, 1 mM sodium fluoride, 1µg/mL aprotinin, 10 µg/mL leupeptin and 1 mM PMSF]. After 15 min centrifugation the cleared lysates were immunoprecipitated and resolved by reduced SDS–polyacrylamide gel electrophoresis, the blots were incubated with the indicated antibodies and the bands were visualized with ECL

PTP Activity

Cells were lysed in cell lysis buffer (20mM HEPES pH 7.7, 2.5 mM $MgCl_2$, 0.1mM EDTA, 1% NP40, 1 mM 4-(2-amino-ethyl)-benzenesulfonylfluoride hydrochloride, 1mM DTT, 10 mg/mL aprotinin, and 10 mg/mL leupeptin). After 15 min of centrifugation, the cleared lysates were immunoprecipitated with GammaBind G Sepharose (GE Healthcare, Diegem, Belgie) and the phosphatase activity was determined using the PTP no radioactive assay kit from Sigma.

Flow Cytometry for apoptosis analysis

To perform the Annexin and PI assay, the FITC Annexin V Apoptosis Detection Kit II from BD Biosciences (San Diego, CA, USA) was used. Briefly, control and apigenin-treated cells were collected, washed twice in cold PBS, ressuspended in binding buffer (provided in the kit) and stained with Annexin V FITC and PI.

Flow Cytometry for Cell Cycle Analysis

After treatment with apigenin, cells were spun down, washed with PBS and ressuspended in work solution (0.96 g/mL sodium citrate, 1 mg/mL Ribonuclease A, 0.02 mg/mL propidium iodide, 0.01% Triton X-100). After incubation in the dark for 60 min at room temperature, the samples were analyzed using FACSCalibur flow cytometer (BD Bioscience).

Electron Microscopy (EM)

HL60 and TF1 treated or non-treated with apigenin for different periods were washed in RPMI, pelleted, and subsequently fixed in 2 %

glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4°C. Cells were dehydrated, osmicated, and embedded in Epon812 according to routine procedures [35]. Semithin sections (1 to 0.5 μm) were examined by electron microscope (Philips 201; Philips, Eindhoven, The Netherlands).

Statistical analysis

All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Cell viability data were expressed as the means ± standard errors of 3 independent experiments carried out in triplicates. Data from each assay were statistically analyzed by ANOVA. Differences were considered significant when the p value was less than 0.05. Western blottings represent 3 independent experiments.

RESULTS

Apigenin as a food-based anti-leukemia compound

Leukemia cells and human peripheral blood lymphocytes were treated with apigenin (Figure 1A) in concentrations up to 200 μM, for 24h. As shown in Figure 1B, apigenin caused a dose-dependent reduction in viable leukemia cells. IC50 of apigenin on HL60 was 30 μM, with complete loss of viability at 100 μM. In contrast, erythroleukemic cells were more resistant, 60% ± 6 reduction of viability at 100 μM was observed for both K562 and TF1 cell lines. Importantly, no strong effect of apigenin treatment was observed on normal human peripheral blood lymphocytes, only 20% of cells were sensitive to apigenin at the highest concentrations. These results indicate that apigenin shows promise as a food-based anti-leukemia compound.

Apigenin reduces survival due to cell cycle arrest

As HL60 and TF1 cells showed more sensitivity toward apigenin, we continued our studies with these two cell lines. We further investigated whether the reduced cell numbers were a result of reduced cell cycling or increased cell death, by following the number of viable cells over the hours using vital stain Trypan Blue. Apigenin 20, 30 and 40 μM reduced to 60.4%±11.7, 79.4%±15.5 and 55.8%±7, respectively, the number of viable HL60 cells (figure 1C) while the non treated cells showed 170% of viability, indicating that apigenin both stops cell proliferation and drives HL60 cells into cell death. TF1 cells showed 175%, 125% and 120% of viable cells at 24h treatment with 25, 50 and 100 μM of apigenin respectively, while non-treated cells showed 225% viability (figure 1D). Even after 36 h of 50 and 100 μM apigenin, the cell viability remained the same as control at 0h, and the base line on 100% was not passed (figure 1D) indicating that apigenin did not induce cell death in TF1 cells, but blocks cell proliferation.

Through analysis of DNA content by flow cytometry, we observed cell cycle arrest of HL60 cells in G2/M phase by apigenin (Figure 2A), as the number of cells in G2/M phase increased dose dependently whereas the number of cells in S-phase decreased. At 100 μM apigenin there were 58%±8 cells in G2/M phases compared to 11.2%±0.6 in non-treated cells ($p<0.05$). The percentage of cells in S phase dropped from 42.3%±1.3 in non-treated cells to 21.4%±2 in treated cells ($p<0.01$). This was in agreement with an increase in apigenin-induced phosphorylation of Cdc2 at Tyr15 which results

in inhibition of this kinase (Figure 2 C). Moreover, the expression of c-myc and p21 remained unchanged (Figure 2 C).

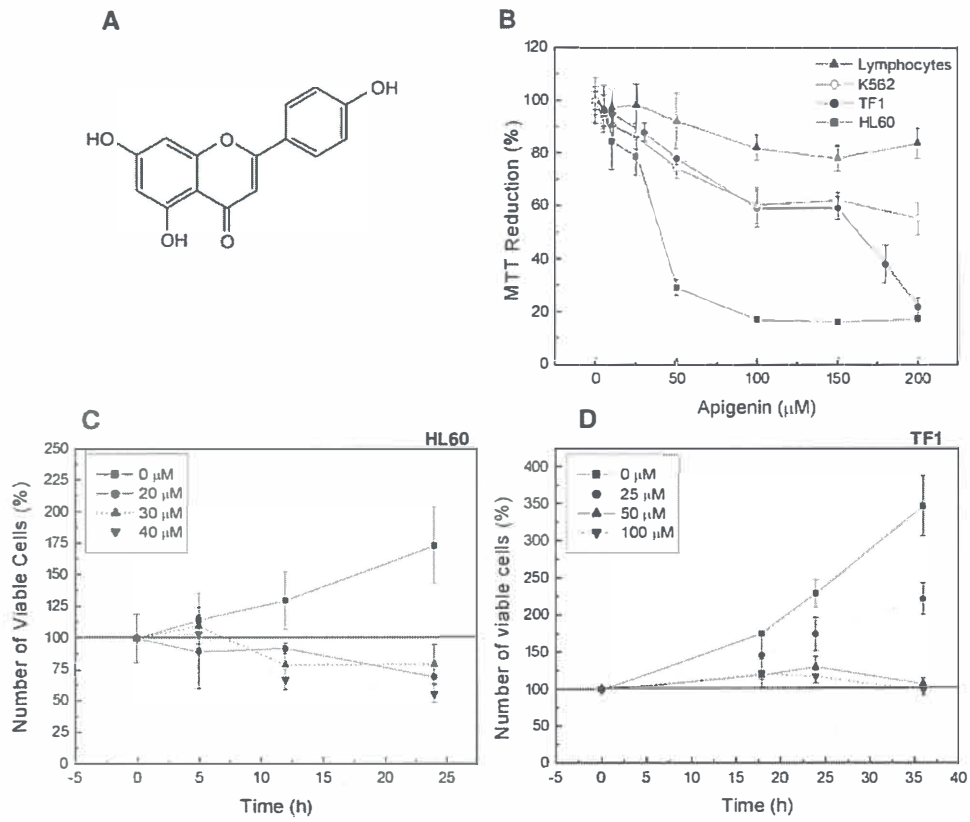

Figure 1. Apigenin reduces cell viability of leukemia cells. **(A)** Molecular structure of apigenin. **(B)** Leukemia cells and human peripheral blood lymphocytes were treated with apigenin for 24 h. Cell viability was measured by MTT assay and cell viability of non-treated cells was considered as 100%. **(C)** Number of HL60 viable cells during different times of treatment with apigenin. After HL60 cells treatment with apigenin for 5, 12 and 24 h, the number of viable cells were quantified using Trypan Blue vital dye. **(D)** Number of TF1 viable cells during different times of treatment with apigenin. TF1 cells were treated with apigenin for 18, 24 and 36 h and using the Trypan Blue vital dye the cell viable was quantified.

TF1 cells were arrested in the G0/G1 phase as shown in Figure 2 B. The number of cells in G0/G1 phase increased from 40.7%±0.34 cells in untreated cells to 65.12%±1.11 upon treatment with 50 μM apigenin ($p<0.01$). The number of cells in S-phase was 44.6%±3.6 in non-treated cells and

decreased to 18.6%±0.9 in cells, which received 50 μM apigenin. Corroborating these data, results shown in Figure 2D revealed that apigenin led to a complete degradation or inhibition of expression of CDK6, a protein essential for passage through the G1/S phase restriction point. However, no changes were observed in CDK4 protein levels. Hence apigenin is a potent inhibitor of cell cycle progression in a variety of model systems of leukemia growth.

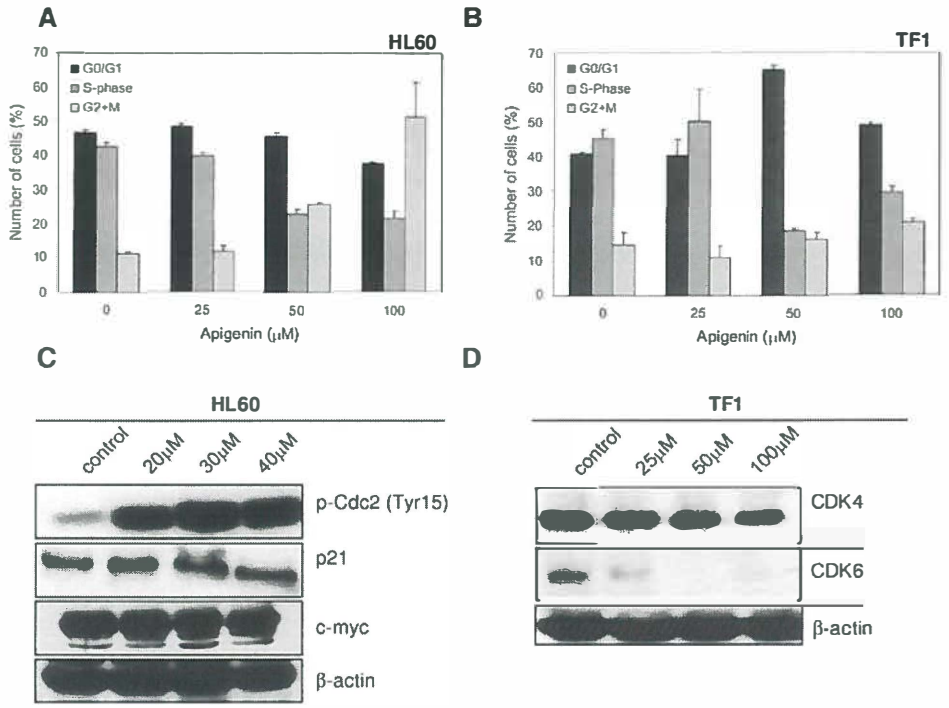

Figure 2. Apigenin induces cell cycle arrest. (A) Number of HL60 cells in different phases of cell cycle (G0/G1 phase, S-phase and G2/M) as determined by propidium iodide staining followed by flow cytometry. **(B)** Number of TF1 cells in different phases of cell cycle. Cell cycle analysis was performed by measuring DNA content by propidium iodide staining after treatment with specified concentrations of apigenin for 24 h. **(C)** Expression of p21 and c-myc (middle and lower panel) and phosphorylation of CDC2 (upper panel), key-proteins in cell cycle control in HL60 cells was determined by western blotting. **(D)** Expression CDK 4 and 6 in TF1 cells by western blotting. Equal loading was confirmed by reprobing blots for β-actin

Downregulation of JAK/STAT pathway in both cells and PI3K/PKB only in HL60 cells by apigenin

We decided to establish the molecular details by which apigenin induces cell cycle arrest through the analysis of phosphorylation status of some kinases and phosphatases involved in leukemia cell proliferation and survival. Figure 3A shows that treatment of HL60 cells with apigenin resulted in downregulation of PI3K/PKB pathway, possibly due to a decrease in PI3Kp85 subunit expression. We also observed diminished phosphorylation of PKB Ser473 and of PDK-1 Ser241. In agreement with diminished PKB activity, we observed diminished GSK3-β Ser9 phosphorylation. Inhibition of the PI3K/PKB pathway can be a direct consequence of activation of PTEN. As shown in figure 3C, apigenin treatment decreases phosphorylation of PTEN at Ser380, leading to its activation, providing a mechanistic explanation for this action of apigenin.

In contrast, no effect was observed on PI3K/PKB pathway in TF1 cells treated with apigenin for 24 h (figure 3B). Phosphorylation levels of PKB at Ser473 and phosphorylation levels of GSK3-β remained unchanged. Unlike HL60 cells, apigenin did not induce activation of PTEN in TF1 cells, which remained strongly phosphorylated at Ser380 (Figure 3C). Thus, apigenin has differential effects depending on the cell type involved.

To obtain more information about the action of apigenin in cell survival, we analyzed MAPKp38, JAK2 and STAT3 and 5. In HL60 cells treated with apigenin an increase in phosphorylation of MAPKp38 was observed at Thr180/182, essential for p38 catalytic action. However, in TF1 cells, the p38 activity remained unchanged after apigenin treatment (Figure 3A). JAK/STAT pathway was downregulated in both cell lines, thus emerging as a general effect of apigenin in leukemia. Apigenin led to decreased phosphorylation of JAK2 and STAT3 in both cell lines and STAT5 in TF1 cells (figure 3A and B). The strong negative effect of apigenin on STAT3 phosphorylation in TF1 cells can be explained by increase of expression (figure 3C) and activity of LMWPTP (figure 3D), one of negative modulators of STATs, as well as the strong inhibition of SHP-2; LMWPTP activity was about 4-fold (385%±94), 2-fold (198%± 28) and 10-fold higher (1083%±47) in the presence of 25, 50 or 100 μM apigenin, respectively. In HL60 cells, the LMWPTP activity was inhibited about 44, 37 and 36% at 20, 30 and 40 μM apigenin, respectively. Another activator of STATs, the Src protein kinase, was also inhibited by apigenin treatment, displaying decrease of phosphorylation at Tyr416, an activation site, in both cell lines, HL60 (figure

3A) and TF1 (figure 3B). Hence, this general response towards apigenin across multiple types of leukemia may be important for explaining its broad anti-leukemia effects.

Both cell lines treated with apigenin displayed a decrease of phosphorylation of SHP2 at activator residue Tyr542 (Figure 3C), which thus may also contribute to the growth inhibition of leukemic cells observed.

TF1 cells display a constitutive activation of mTOR and, consequently, activation of protein synthesis/cell growth pathways (figure 3E). In the presence of apigenin we observed downregulation of p70S6K phosphorylation and consequently, of S6 protein in TF1 cell. HL60 cells, however, do not show constitutive activation of mTOR and p70S6K, although apigenin decreased S6 protein phosphorylation. Therefore, the functional importance of mTOR pathway regulation in the apigenin response remains unclear.

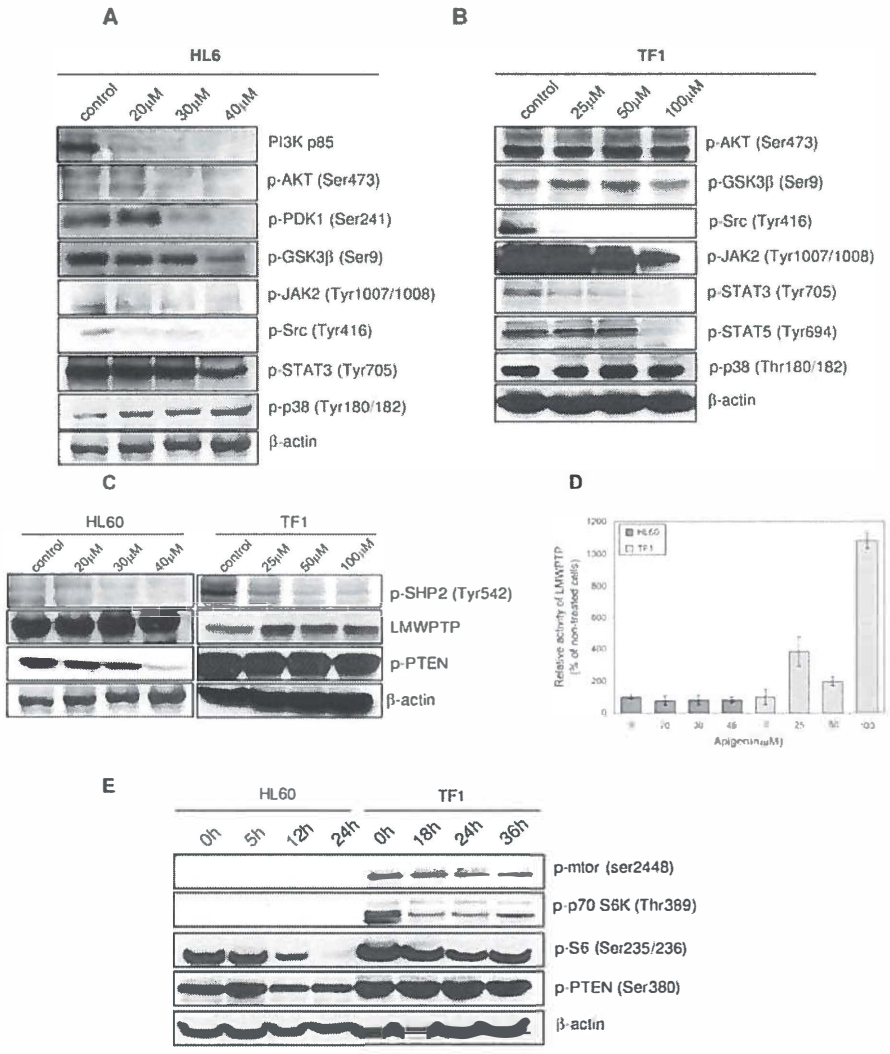

Figure 3. Analysis of cell proliferation pathways. Effect of apigenin treatment on key kinases involved in cell proliferation from HL60 **(A)** and TF1 **(B)** cells. **(C)** Effect of apigenin treatment on phosphatases involved in proliferation of HL60 and TF1 cells. Cells were treated with specified concentrations of apigenin for 24 h and the expression or phosphorylation of the proteins was determined by western blot. Equal loading was confirmed by reprobing blots for β-actin. **(D)** The activity of immunoprecipitated LMWPTP was measured after apigenin treatment of HL60 and TF1 cells by Malachite green assay. **(E)** Effect of apigenin treatment on proteins involved in cell survival control. HL60 cells were treated with 50 μM apigenin for 5, 12 and 24 h and TF1 cells were treated with 100 μM apigenin for 18, 24 and 36 h and the phosphorylation of the proteins was determined by western blotting. Equal loading was confirmed by reprobing them for β-actin.

Apigenin induces caspase-dependent apoptosis in HL60, but not in TF1 cells

In HL60, we observed a significant increase in annexin V positive cells, 31.9%±4.1 and 48.13%±2.6 for 50 and 100 μM apigenin, respectively (figure 4A). Consistent with being more resistant to apigenin, only moderate induction of apoptosis was observed at high concentrations of apigenin in TF1 cells; at 100 μM, 14.5%±0.1 of cells stained annexin V positive ($p<0.002$).

To obtain more information about apigenin-induced apoptosis in HL60 and TF1 cells, we pre-treated both lineages for 1 h with 50 μM of Z-VADfmk, a pan-caspase inhibitor, prior to the 24 h apigenin treatment. Figure 3 B shows that pre-treatment of HL60 cells with Z-VADfmk reduced the number of apoptotic cells 1.3 fold when compared to cells which received apigenin only. No significant difference in apoptosis induction was observed in TF1 cells pre-treated with Z-VADfmk (figure 4B), suggesting that apigenin induces caspase-independent apoptosis in TF1 cells and caspase-dependent apoptosis in HL60 cells.

To understand the role of the PI3K/PKB pathway in apigenin-induced apoptosis, we treated cells for 2 h with 5 μM of LY294002, a PI3K inhibitor, prior to the 24h apigenin treatment. As shown in figure 4C, inhibition of PI3K with LY294002 prior to apigenin treatment increased the number of apoptotic HL60 cells 1.9 fold when compared to cells which received apigenin only, showing the importance of PI3K/PKB pathways in apoptosis resistance in this lineage. No statistic difference in apoptosis induction was observed in TF1 which received LY294002 prior to apigenin treatment comparing to cells which received apigenin only (figure 4D), demonstrating that TF1 cells are not dependent on PI3K/PKB pathway activation for their resistance to apigenin induced apoptosis.

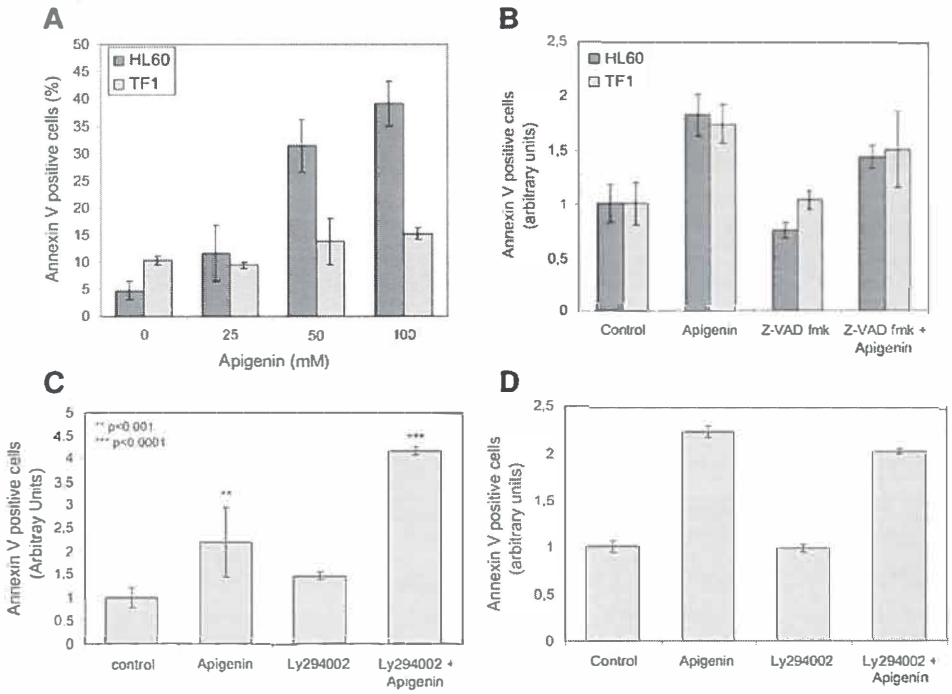

Figure 4. Analysis of apigenin-induced apoptosis in HL60 and TF1 cells. (**A**) Percentage of apoptotic cells after 24 h treatment with apigenin (25, 50 and 100 μM) was determined by flow cytometric analysis of Annexin V staining. (**B**) The effect of pan-caspase inhibitor Z-VADfmk on apigenin-induced cell death. Cells were pre-treated with 50 μM Z-VADfmk for 1 hour prior to apigenin treatment. HL60 cells were treated with 50 μM apigenin and TF1 cells were treated with 100μM apigenin for 24 h. (**C** and **D**) Effect of PI3K inhibitor Ly294002 on apoptosis induction in HL60 (**C**) and TF1 (**D**) cells. Cells were pre-treated with 5 μM Ly294002 for 1 hour prior to 24 h apigenin treatment. Afterwards, cells were stained with Pi and Annexin V. The combined Annexin V positive fractions were considered apoptotic. (*** $p<0.001$, ** <0.01)

Molecular mechanism of apoptosis induction by apigenin

To further elucidate the apigenin induced cell death mechanisms in HL60 and TF1 cells, we analyzed the levels of cleaved caspase 3, 7, 8 and PARP. We observed an increased dose (figure 5A) and time dependent effect (figure 5C) on cleaved caspase 3 levels in HL60 cells. This response was not observed in TF1 cells; only after treatment with 100 μM apigenin a small amount of cleaved caspase 3 (17kDa fragment) became apparent (figure 5C). In

HL60 cell, caspase 8 cleavage in apigenin treated cells was observed, indicating that the apoptosis extrinsic pathway was also involved in apigenin cell death induction in this cell line (figure 5B). In HL60 the levels of full length caspase 7 decreased when cells were treated with 50 μM apigenin, coinciding with increased presence of the 20kDa cleaved caspase 7 fragment (figure 5C).

In TF1 cells, neither cleavage of caspase 8 nor caspase 7 was observed (figure 5A and B). Furthermore, the levels of cleaved PARP remained unchanged in apigenin treated cells. We observed an increased of AIF levels at 25 and 50 μM of apigenin treatment in TF1 cells (figure 5A), a strong indication of caspase-independent apoptosis. These results emphasize that despite the generalized leukemia growth inhibition observed, the molecular consequences of apigenin treatment show substantial context dependency.

Apigenin and autophagy

To obtain more insight into the apigenin response in TF1 cells, we analyzed the apoptosis and autophagy pathway activation in the time domain. For apoptosis induction we used TNFα (10 ng/mL) as a control. TF1 cells were slightly induced to undergo apoptosis by TNFα, as was deduced from cytokine induced cleavage of caspase 7 to its 30kDa, but not 20kDa fragment and PARP cleavage. Interestingly, TNFα also induced cleavage of LC3BI into LC3BII after 24 and 36 h of treatment and also induced high expression of the autophagic proteins Atg5 and 12 (figure 6B). In addition, through EM we could observe the signature double membrane vacuoles, generally accepted as strongly indicative for autophagy (figure 6 D, F). In addition, TNFα activated P70S6K which led to high phosphorylation of S6 protein, indicative for protein synthesis (figure 6C), and apparently confirmed by EM, as TNFα-treated TF1 cells showed high numbers of ribosomes on endoplasmic reticulum (ER) surface, indicative of high ER activity (Figure 6E). Apigenin also induced autophagy in TF1 cells, but through inhibition of mTOR and P70S6K. We observed a strong reduction on phosphorylation of S6 protein. No changes in beclin -1 levels were observed, however there was a strong reduction of Atg5, 7 and 12 on TF1 cells treated with apigenin. These data led us to conclude that autophagy is a dominant response to apigenin treatment in at least some types of leukemia

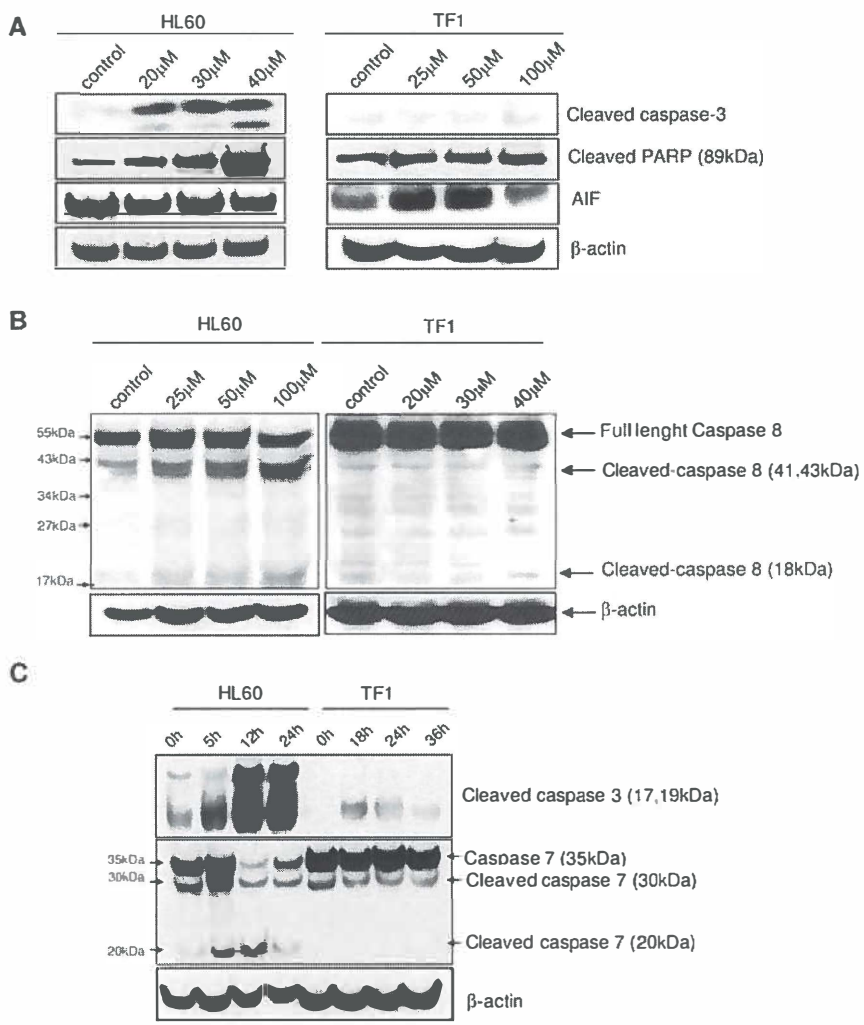

Figure 5. Analysis of apoptosis pathway mechanism. (A) HL60 and TF1 cells were treated with apigenin for 24 h. The expression of cleaved caspase 3, cleaved PARP and AIF was evaluated by immunoblotting. (**B**) Levels of full length and cleaved caspase 8 (41, 43 and 18kDa.) were determined. (**C**) Levels of cleaved and uncleaved caspase 7 and cleaved caspase at different periods of apigenin treatment was investigated. HL60 cells were treated with 50μM apigenin for 5, 12 and 24 h and TF1 cells were treated with 100 μM apigenin for 18, 24 and 36 h. Soluble lysates were matched for protein content and analyzed by Western blot analysis. Equal loading was confirmed by reprobing the blots with anti-β-actin antibodies.

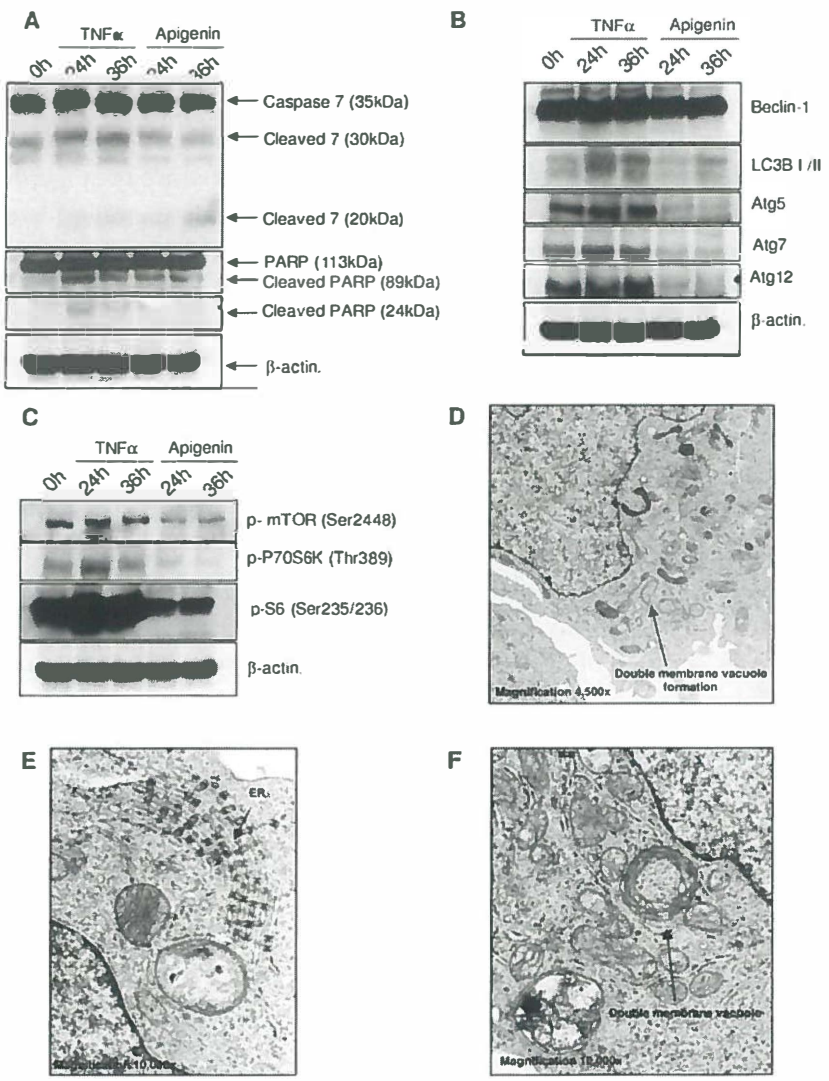

Figure 6. Effect of apigenin treatment on key kinase molecules involved in the autophagy and apoptosis from TF1 cells. TF1 cells were treated with 10 ng/mL TNFα or 100 μM apigenin for 24 and 36 h. (**A**) Levels of full length and cleaved caspase 7 (30 and 20kDa), full length and cleaved PARP. (**B**) Levels autophagy proteins beclin-1, LC3B, Atg5, 7 and 12. (**C**) Analysis of P70S6K/S6 pathway by western blotting. The expression or phosphorylation of the proteins was determined by western blot analysis. Equal loading was confirmed by reprobing them for β-actin. (**D**) Electron microscopy of TF1 cells treated with TNFα for 24 h, detail of double-membrane vacuole in formation (magnification 4,500x). **(E)** Detail of endoplasmic reticulum, showing intense protein synthesis (magnification 10,000x). (**F**) Detail of double-membrane vacuole (magnification 10,000x).

Apigenin leads to apoptosis-like cell death in HL60 and involves autophagy in TF1 as revealed by EM

Further analysis of apigenin effects on HL60 and TF1 cells was performed using EM, emphasizing the differential nature of apigenin responses in leukemia (figure 7, 8). In HL60 cells treated with apigenin for 8 hours, we observed classical apoptosis structures, chromatin condensation and reduction of cell volume (figure 7B). At 24 h of treatment most of cells showed electron dense cytoplasm with very condensed nucleus and many of non electron dense vacuoles (figure 7C), and some necrotic cells were detected (figure 7C). In the electron-dense-cytosol-cell we identified double-membrane vacuoles, as shown in detail in figure 7 D. TF1 cells showed morphological changes only after 36 h of treatment, where we could find speckles in the nucleus (arrows on the figure 8C) and electron dense mitochondria in the cytoplasm (figure 8C). In addition, a number of cells displayed non electron dense vacuoles and double membrane vacuoles, strong evidence of autophagy (figure 8D). Thus there is not a common pathway leading to leukemia cell death, despite the general anti-leukemia activity of apigenin and experiments were initiated to explore the functional consequences of this observation.

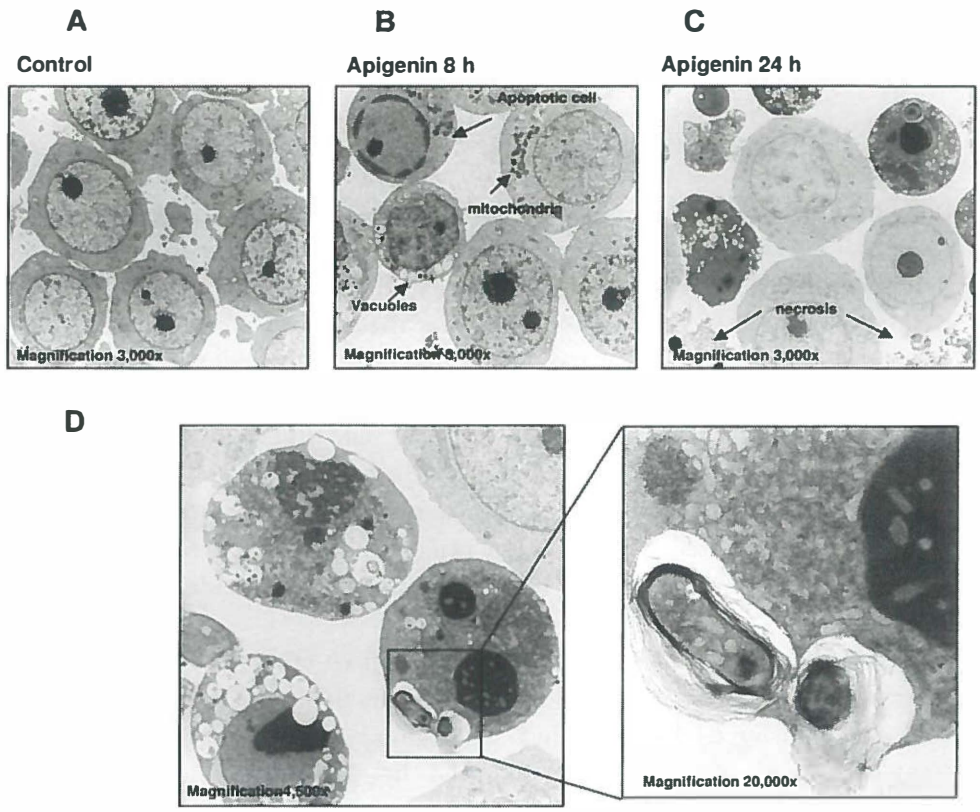

Figure 7. Electron microscopy of HL60 cells treated with apigenin for different time points. (A) Control cells, without treatment (magnification 3,000x). **(B)** Cells treated with apigenin for 8 h, displaying classical apoptotic cells (condensed chromatin, reduction of cell size and no vacuoles); cells with dense mitochondria and no condensed chromatin and cells with size reduction, many vacuoles and no condensed chromatin (magnification 3,000x). **(C)** HL60 cell treated for 24 h (3,000x), showing necrotic cells and cells with many vacuoles. **(D)** Cells treated for 24 h with apigenin, displaying cells with dense chromatin, many vacuoles and double membrane, detail in high magnification (20,000x) of vacuoles with cytosol content.

Apigenin diminishes the cytotoxic effect of the chemotherapeutic vincristine in TF1 cells

The role of autophagy in cells can be of dual consequence either it can prolong cell survival in the absence of nutrients or can result in cell death. Induction of autophagy is often associated with chemotherapy resistance. Indeed, TF1 cells pretreated with apigenin 24h prior of vincristine showed diminishment of chemotherapic cytotoxic effect (figure 8E, F). TF1 cells were treated with 100 μM apigenin for 24 h, after the number of cells of treated and non-treated cells was equalized, and cells were cultured with or without vincristine treatment for an additional 24 h. After the treatment, the viable cells were counting using the vital dye trypan blue (figure 8E). In the absence of apigenin pre-treatment, vincristine reduced the number of viable to 39.2%±6.1, compared to cells which did not receive vincristine (100%). However, cells treated with apigenin prior to vincristine treatment showed 147%±7.3 of viability, compared to the cells which did not received vincristine (100%).

To obtain more data about the protective effect of apigenin against vincristine, we further analyzed apoptosis induction. TF1 cells were treated with apigenin for 24 h, after which the number of cells of treated and non-treated cultures was equalized followed by the presence or absence of vincristine treatment for an additional 24 h. After the treatment, we analyzed apoptosis induction using flow cytometric analysis of annexin V binding (figure 8F). Cells which received neither apigenin nor vincristine showed 10.7%±0.007 annexin V positive cells. Vincristine treatment alone induced annexin V staining in 33.6%±1.4 of cells. Treatment of cells with apigenin alone (for a total of 48h) displayed 22.34%±0.4 annexin V positive cells. Strikingly, cells that were pretreated with apigenin followed by vincristine treatment showed a reduction in annexin V positive cells when compared to vincristine treatment alone (19.8%±0.2 of cells) (figure 8F). Thus we showed that apigenin protects TF1 against cell death induced by vincristine. We conclude that although the effects of apigenin are generally chemopreventive with regard to leukemia disease, differential response may include resistance to chemotherapy. Thus, the use of such food-based compound as an adjuvant setting when disease is established might not be advisable.

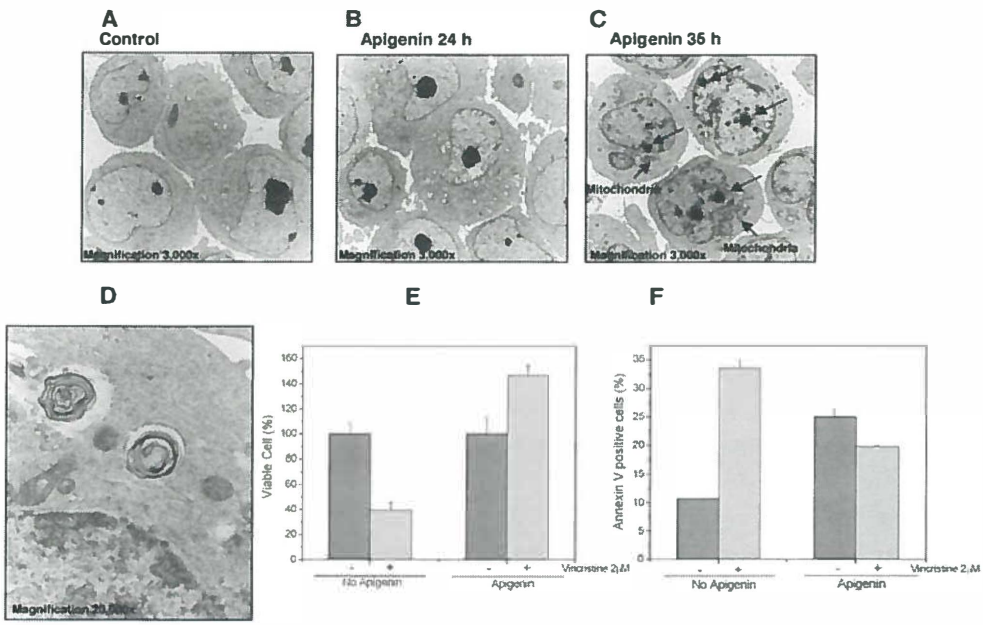

Figure 8. Electron microscopy of TF1 cells treated with apigenin for different time points. (A) Control cells, without treatment (magnification 3,000x). **(B)** Cells treated with apigenin for 24 h, display little morphological difference from non-treated cells (magnification 3,000x). **(C)** TF1 cells treated for 36 h (magnification 3,000x) present dense mitochondria, partly condensed chromatin and speckles (arrows) (magnification 3,000x). **(D)** Detail of vacuoles with double membrane in an authophagic cell (magnification 20,000x) (**E**) Number of viable TF1 cells after vincristine treatment with ou without apigenin pre-treatment. TF1 cells were treated or not treated with 100 μM apigenin for 24h, after which cell numbers were normalized and cultured for another 24 hours with or without 2 μM vincristine. The number of viable cell was determined using Trypan Blue vital dye. (**F**) Determination of percentage of apoptotic TF1 cells by flow cytometric analysis of Annexin V staining. Cells were treated or not treated with apigenin for 24 h, after which cell numbers were normalized and cells were cultured for another 24 hours with or without 2 μM vincristine.

DISCUSSION

Our research group has a long-standing interest in the possible beneficial biological effects of natural compounds and their derivatives on malignant disease [10-14]. In this work, we explored the effects of apigenin in leukemia, flavonoid common present in human diet, which is emerging not only as potent cancer preventing agents, but also useful for adjuvant therapy [11]. Although the induction of apoptosis and cell cycle arrest, and inhibition of cancer-driving signaling pathways certainly supports the chemopreventive action, especially as untransformed lymphocytes do not seem to be really affected by the compound, the observed interaction with therapeutically relevant drugs seems to rule this compound out for adjuvant therapy as it may actually hamper treatment of established disease.

In HL60 cells, apigenin induced cell cycle arrest in G2/M, whereas TF1 cells were arrested in the G0/G1 phase. The control of cell cycle is mediated by many protein complexes such as CDK and cyclins. Cdc2, normally driving cells into mitosis, is the ultimate target of pathways that mediate rapid arrest in G2, in response to DNA damage [15]. CDK6 forms a complex with cyclin-D and targets the retinoblastoma protein, allowing passage through the G1/S phase restriction point [16]. Many studies have shown that apigenin blocks cell cycle in cancer cells (Choi & Kim, 2009; Lepley, 1997), however, depending on the cell type, apigenin can arrest the cell cycle in different phases, for example, it can lead G2/M arrest in colon carcinoma [2], breast cancer and pancreatic cancer cells [19]. Nevertheless, in human prostate cancer, LNCap and PC3 cells, apigenin induced cell cycle arrest in G0/G1 [20]. Our current study extends these observations that, depending on the cyclins affected, even in the leukemic compartment responses may be substantially different. The outcome of cell cycle arrest may depend on the phase in which the cell is arrested. Numerous studies, including those using dietary compounds, have linked G2/M arrest with subsequent apoptosis [21,22], as was observed for HL60 cells in our study. In contrast, G0/G1 arrest is seen upon reduction of mTOR activity [23], and has been associated with induction of autophagy [24], reminiscent of apigenin effects in TF1 cells in the current study. p70S6K/S6 signaling has been implicated in inhibition of autophagy [25] and inactivation of the mTOR signaling pathway is a prominent consequence of PTEN activation [26]. In the present study, although apigenin was not able to activate PTEN in TF1 cells, mTOR was strongly downregulated by apigenin. Thus this flavonoid may be an attractive therapeutic agent for treating tumors carrying inactivated PTEN. Studies with

cancer stem cells have shown that inhibition of mTOR pathways by rapamycin can block the generation or maintenance of leukemia-initiating cells in the PTEN-deficient mice [27].

The role of autophagy in cell survival is a dual one; it can either help cells to survive during stress or end in apoptosis in the absence of cell rescue [28]. The autophagic characteristics observed in HL60 cells suggest that the latter predominates in this cell type following exposure to apigenin, whereas in TF1 cells autophagy is not followed by apoptosis, in apparent agreement with the therapy resistant phenotype of these cells. Only after 36 hours of stringent apigenin treatment did a small fraction of TF1 cells show signs of (a caspase-independent form of) apoptosis. Interestingly, while showing hallmarks of apoptosis upon TNFα treatment, e.g. cleavage of PARP and caspase activation, EM analysis of TNFα-treated cells demonstrated characteristics of autophagy, confirmed by high levels of sentinel autophagic proteins beclin-1, LC3BII, Atg5, 7 and 12. In addition, TNFα induced activation of the p70S6K/S6 pathway, indicating that the induction of autophagy in TNFα treated cells is induced by high expression of autophagic genes related to vacuoles, rather as of inhibition of P70S6K/S6 pathway.

Flavonoids have the potential to bind to the ATP-binding sites of a large number of proteins due to their similarity to ATP structure, which make flavonoids competitive inhibitors of protein kinases [29]. Apigenin had already been reported to be a competitive inhibitor of protein tyrosine kinases [30]. In this work we clearly observed that apigenin inhibited PKB/PI3K pathway in HL60 cells, through activation of PTEN and downregulation of PDK1 and PI3K. Unlike HL60, TF1 showed no changes in PI3K/PKB pathway under apigenin treatment. Furthermore, MAPKp38 was activated in HL60 cells treated with apigenin, but not in TF1 cells. Recently it has been suggested that p38 and JNK may be involved in the apoptotic process by repression of ERK pathways [31]. Thus, the differential effects of apigenin observed on leukemia cell cycle are reflected by differential effects on kinase activities.

Interestingly, the only common target of apigenin in both types of leukemia was the c-Src/JAK/STAT pathway, which constitutes a major mediator of cytokine activity. Constitutively active JAK2 proteins are associated with myeloproliferative disorders, acute lymphoblastic leukemia, acute myeloid leukemia and acute megakaryoblastic leukemia, and inhibition of STAT3 leads to increased apoptosis, decreased proliferation, and decreased tumor size [32]. JAK/STAT pathway is regulated by SHP2, which can stabilize JAK2 protein or inducing Src kinase activation. Constitutive

activation of SHP2 in mice cooperates with progression of myeloproliferative disorders [33]. In the present work we observed that apigenin downregulated SHP2 and thereby JAK2, which led to a decreased phosphorylation and activation of STAT3 and 5. This effect was more apparent in TF1 cells, which can be explained by the increased level and activity of LMWPTP, another JAK/STAT downregulator, in this cell line. Further experimentation should reveal whether the JAK/STAT signaling cassette really is the principal target for apigenin effect in cancer in general.

Because apigenin induced cell cycle arrest and autophagy in TF1 cells, we hypothesized that apigenin could inhibit the action of chemotherapeutic agents against leukemic cells. One of the most common chemotherapeutics is vincristine, and indeed upon apigenin treatment, cells became more resistant to vincristine-induced cell death. Vincristine binds to tubulin dimers, inhibiting the formation of microtubules. Disruption of the microtubules arrests mitosis in metaphase, therefore, apigenin-induced blockage of TF1 cells in G0/G1 phase might enable these cells to escape the action of vincristine. This action of apigenin clearly disqualifies this compound for adjuvant strategies once disease is established, at least in relation to vincristine. However, it is important to mention that when we combined apigenin with imatinib or mitoxantrone no difference was observed (unpublished data). Therefore, this observation reinforces our hypothesis that the undesirable action of apigenin in presence of vincristine might be related to the action mechanism of chemotherapic.

In conclusion, apigenin is a potential chemopreventive and chemotherapeutic agent in leukemia due to the stimulation on signaling pathways that provoke inhibition of cell proliferation and cell cycle arrest of fast-cycling cells. This work emphasizes the importance of a deep analysis of molecular mechanism and biochemical details modified by a natural compound with clinically useful properties. Extreme care with respect to consumption of such apigenin-like compounds during chemotherapy must be exercised to prevent compromising clinical success, and trails using such compound should not be frivolously initiated. However, the possibility of using apigenin as a model for drug design should not be discarded.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grant Proc. 2004/15041-5 from FAPESP and by Grant T3-103 from TI Pharma. Scholarship to RRRS was provided by CAPES and CNPq.

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Chapter 6

Ferruginol supress survival signaling pathways in androgen-independent human protaste cancer cells

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Biochimie. 2008, 90(6): 843-54

ABSTRACT

Ferruginol, a bioactive compound isolated from a Chilean tree (Podocarpaceae), attracts attention as a consequence of its pharmacological properties, which include anti-fungal, anti-bacterial, cardioprotective, anti-oxidative, anti-plasmodial and anti-ulcerogenic actions. Nevertheless, the molecular basis for these actions remains only partly understood and hence we investigated the effects of ferruginol on androgen-independent human prostate cancer cells (PC3), a known model for solid tumor cells with an exceptional resistance to therapy. The results show that ferruginol induces PC3 cell death via activation of caspases as well as apoptosis-inducing factor (AIF) as confirmed by its translocation into the nucleus. In order to clarify the biochemical mechanism responsible for the anti-tumor activity of ferruginol, we analyzed a set of molecular mediators involved in tumor cell survival, progression and aggressiveness. Ferruginol was able to trigger inhibition/downregulation of Ras/PI3K, STAT 3/5, protein tyrosine phosphatase and protein kinases related to cell cycle regulation. Importantly, the toxic effect of ferruginol was dramatically impeded in a more reducing environment, which indicates that at least in part, the anti-tumoral activity of ferruginol might be related to redox status modulation. This study supports further examination of ferruginol as a potential agent for both the prevention and treatment of prostate cancer.

1. INTRODUCTION

Prostate cancer is a major cause of cancer-related death among males and the second leading cause of cancer death in Western countries. Although recent years have seen an improvement in prostate cancer diagnosis, only a few novel therapeutic strategies have emerged and there has been little progress in improving survival [1,2]. Therefore, novel strategies for dealing with this disease are called for. Our research group has a long-standing interest in the possible beneficial biological effects of natural compounds and/or their derivatives, such as antioxidants [3] and anti-tumor agents [4e10], and hence we were interested whether we could define novel compounds with therapeutic potential for prostate cancer. Among the different classes of natural compounds, the diterpenoids have been shown to present a potent anti-proliferative action [4,11]. Ferruginol, an active compound isolated from the Chilean tree *Persea nubigena* and from the stem bark of *Podocarpus andina* (Podocarpaceae), is an abietane diterpene occurring in plants belonging to the Podocarpaceae, Cupressaceae, Lamiaceae and Verbenaceae families. This diterpene presents promising biological activities, such as anti-fungal and anti-bacterial [12], miticidal [13], cardioactive [14], anti-oxidative [15], anti-plasmodial [16] and anti-ulcerogenic [17] properties.

We decided to investigate the potential effects of ferruginol in prostate cancer. In this work we show for the first time the molecular mechanism by which ferruginol, induces resistant prostate cancer cell death. Ferruginol was able to trigger inhibition/ downregulation of Ras/PI3K, STAT 3/5, protein tyrosine phosphatase and protein kinases related to cell cycle regulation. Importantly, the toxic effect of ferruginol was dramatically impeded under the condition of more reducing environment, which indicates that at least in part, the anti-tumoral activity of ferruginol might be related to redox status modulation.

This study supports further examination of ferruginol as a potential agent for both the prevention and treatment of prostate cancer.

Abbreviations: AIF, apoptosis-inducing factor; Bax, Bcl-2 associated x protein; CDKIs, cyclin-dependent kinase inhibitors; CDKs, cyclin-dependent kinases;DISC, death-inducing signaling complex; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated protein kinase 1/2; GSK-3b, glycogen synthase kinase-3b; Hsp27, heat shock protein 27; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p21, tumor suppressor protein; PC3, androgen-independent prostate cancer cell line; PI3K, phosphoinositide 3-kinase; Rb, retinoblastoma; STAT, signal transducer and activator of transcription; TNFR, tumor necrosis factor receptor; FADD, Fas-associated death domain protein; AKT/PKB, protein kinase B; PCNA, proliferating cellular nuclear antigen; NFkB, nuclear factor kappa B; IKK, inhibitory-kappa B kinase; LMWPTP, low molecular weight protein tyrosine phosphatase; JAK, Janus kinase.

2. MATERIAL AND METHODS

2.1. Cell line and reagents

PC3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Ferruginol (Fig. 1A) was extracted from the wood of P. nubigena Lind. and from the stem bark of P. andina (Poepp. ex Endl.) de Laub. (Podocarpaceae) as previously described [18]. Polyclonal antibodies against phospho p38MAPK, phospho- p42/p44 MAPK (ERK1/2) Thr202/204, ERK1/2, phospho- MEK1/2 Ser217/221, pan-AKT, phospho-AKT Ser473, phospho-Hsp27 Ser82, phospho-c-Raf Ser338, phospho- GSK-3b Ser9, AIF, phospho-cdc2 Thr15, phospho-Rb Ser795, phospho-STAT5 Tyr694, phospho-PI3K p85 subunit, CDK6, CDK4, cyclin D1, cyclin D3, PCNA, tubulin, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against p21, NFκB p65 subunit, phospho-STAT3 Tyr705, phospho-STAT3 Ser727, Bcl2, Bax, TNF receptor 1, FADD, IKKa and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LMWPTP antibody was from Abcam. Caspases 3, 8 and 9 Colorimetric Assay Kits were obtained from R&D Systems (Minneapolis, MN).

2.2. Cell culture

PC3 cells were cultured in RPMI containing 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum, at 37°C in a 5% CO_2 humidified atmosphere. In all experiments, cells at semi-confluence were treated for 24 h with different concentrations of ferruginol.

2.3. MTT assay for cellular viability

Cell viability was assessed by MTT reduction assay as previously reported [19,20]. The effect of ferruginol on cell growth was assessed as the percentage of inhibition in cell growth where non-treated cells were taken as 100% of viability. IC_{50} values were determined from three independent experiments.

2.4. Western blotting analysis

Following treatment of cells with ferruginol, the medium was aspirated and the cells were washed with cold physiological solution. The cells

were then incubated in 200 ml of lysis buffer (50 mM TriseHCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate and protease inhibitors (1 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry method [21]. An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100 mM TriseHCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 50 μg of protein, were resolved by SDS polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence ECL.

2.5. Immunoprecipitation of LMWPTP

After treatment of the cells with ferruginol for 24 h, whole-cell lysates were prepared with lysis buffer (20 mM HEPES pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1% Nonidet-P40 (NP40), 1 mM 4-(2-amino-ethyl)-benzenesulfonyl-fluoride hydrochloride), 1 mM DTT, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) and chilled on ice for 2 h. After centrifugation, lysates were rotated with anti-LMWPTP and Protein A-Sepharose at 4°C for 2 h. The beads were washed three times with lysing buffer and twice with 0.5 M Mes, pH 6.0. Next, the phosphatase activity was determined using pNPP as a substrate.

2.6. Caspases 3, 8 and 9 activity assays

Caspase activities were determined by the measurement at 405 nm of p-nitroaniline (pNA) released from the cleavage of Ac-DEVD-pNA, IETD-pNA and LEHD-pNA as substrates of caspases 3, 8 and 9, respectively. The enzyme activities were expressed in pmol/min and the extinction coefficient of pNA was 10,000 M^{-1} cm^{-1}.

2.7. NFκB p65 and AIF nuclear translocation

Briefly, 2 x 10^7 cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.2 ml ice-cold cell extract buffer (10 mM HEPES (N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid) e KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM DTT, and 0.2 mM phenylmethysulfonyl fluoride (PMSF)). The cells were kept on ice for 10 min to allow them to swell, mixed by vortex for 10 s, and microfuged at 4°C at 14,000 x g for 30 s. The supernatant was discarded, and the pellet was resuspended in 30 µl nuclear extraction buffer (20 mM HEPESeKOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 14,000 x g for 2 min. The supernatant was saved as the nuclear extract and used in Western blotting assay.

2.8. Quantification of reduced and oxidized glutathione

Cells were washed twice with PBS, detached with 5 mM EDTA in PBS and washed twice again with cold PBS. Cell number was counted using a hemocytometer; afterwards the cells were centrifuged and equal volumes of cold PBS and 6% 5-sulfosalicylic acid (SSA) were added to cell pellets. The samples were sonicated, centrifuged at 10,000 rpm for 5 min and the supernatants used for the assays. Total glutathione (GSH + GSSG) and glutathione disulfide (GSSG) were determined by using recycling assays involving the reaction of 5,5'-dithio-bis(2nitrobenzoic acid) and glutathione reductase. The total amount of glutathione was calculated from a reduced glutathione standard curve prepared in SSA. For GSSG assay, 100 µl of supernatant was incubated with 2 µl of 2-vinylpiridine for 60 min on ice. The amount of GSSG was calculated from the GSSG standard curve. The amount of reduced GSH per cell was calculated by subtracting the amount of GSSG per cell from the amount of total glutathione per cell [22].

2.9. Annexin V and 7-amino-actinomycin D assays

Control and ferruginol-treated cells were collected and resuspended in 1x binding buffer (0.01 M HEPES-NaOH (pH 7.4), 0.14 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of 2 x 10^7 cells/ml. Subsequently, 100 ml of cell suspension was transferred to a 5 ml tube and 5 µl each of Annexin V-APC

and 7-amino-actinomycin D (7-AAD) was added. Cells were incubated at room temperature for 15 min, after which 400 µl of 1 x binding buffer was added and apoptosis detected by flow cytometry (Becton Dickinson FACSCalibur, Rockville, MD), the data obtained were analyzed using the software Cell Quest Pro BD Biosciences Pharmingen (Erembodegem, Belgium).

2.10. Flow cytometry

After treatment of PC3 cells with ferruginol for 24 h, cells were harvested by the addition of 5 mM EDTA and gently washed off the plate. Cells were pelleted along with the previously collected media. Cell pellets were fixed with 70% ethanol for 30 min on ice and than rinsed three times with 1 ml of 0.1% glucose in PBS (20 mM NaH_2PO_4, 150 mM NaCl), repelleted and resuspended in propidium iodide (PI) staining solution (10 µl of 10 mg/ml RNase A, 5 µl of 10 mg/ml PI per 1 ml of PBS with 0.1% glucose). After 30 min the cells were analyzed using a flow cytometer.

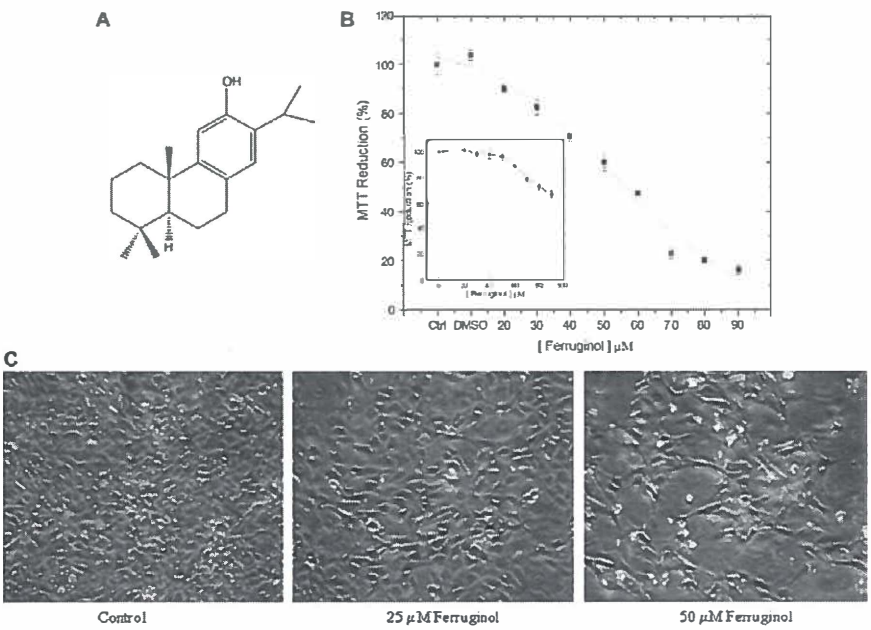
2.11. Statistical analysis

All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Cell viability data were expressed as the mean ± standard error of three independent experiments carried out in triplicate. Data from each assay were analyzed statistically by ANOVA. Differences were considered significant when the P value was less than 0.05. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to b-actin or tubulin ratio).

3. RESULTS

3.1. Inhibition of PC3 growth by ferruginol

PC3 cells were treated with ferruginol in concentrations up to 100 μM and the effect of ferruginol on cell viability was determined employing the MTT method. As shown in Fig. 1B, ferruginol caused a dose-dependent reduction in the cell number displaying an IC_{50} value of 55 μM. Importantly, pre-treatment of the cells with 10 mM GSH prevented the toxic action of ferruginol (inset plot). In agreement, the microscopy analysis also demonstrated a decrease of the cell number (Fig. 1C).



Figue 1. Ferruginol induces toxic effects in PC3 cells. Cells were treated with specified concentrations of ferruginol (A) for 24 h. (B) Mitochondria function was evaluated through the MTT reduction. The results are expressed as the mean % of absorbance (ratio of absorbance in ferruginol treated and control cells); cell viability was also evaluated when the cells were pre-treated for 1 h with 10 mM GSH (inset plot). (C) Morphological analysis of PC3 cells treated with 25 μM and 50 μM ferruginol for 24 h (100 x magnification). Ferruginol was dissolved in DMSO and the final concentration of this solvent was remained at 0.1%.

3.2. Ferruginol induces apoptosis of PC3 cells via caspases and AIF activation

In the next series of experiments, it was determined whether treatment of PC3 cells to ferruginol led to apoptosis. Ferruginol caused around 15% and 30% (in the presence of 25 and 50 μM ferruginol, respectively) of cell death via apoptosis as detected through phosphatidylserine exposure (Fig. 2A). In agreement, we also observed activation of caspases 8, 9 and 3 (Fig. 2B). Additionally, at the lowest concentration, ferruginol led to an overexpression of TNFR1; however, FADD expression was not affected. We also examined the possible participation of mitochondria in response to ferruginol. Bcl2:Bax ratio was not significantly affected; however the expression of AIF was dramatically increased at 50 μM ferruginol. Accordingly, AIF nuclear translocation was detected (Fig. 2C). These findings suggest that PC3 cell response toward ferruginol involves activation of caspases and release of AIF from mitochondrial intermembrane space.

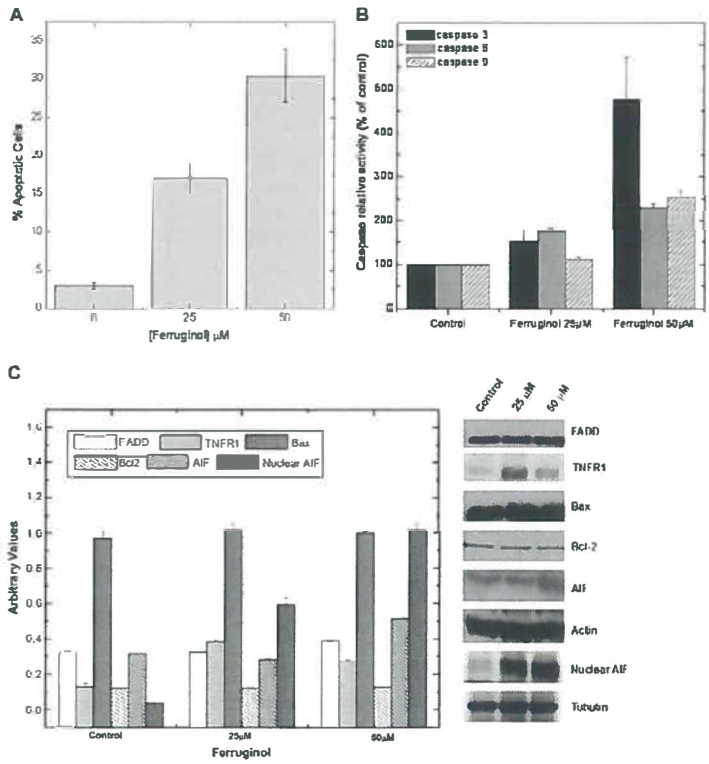


Figure. 2. Apoptosis induction of PC3 cells by ferruginol. (A) Cell samples were prepared as described in Section 2 and Annexin V-positive, 7-AAD-positive and

Annexin V/7-AAD-positive populations were analyzed by flow cytometry. (B) Caspases 3, 8 and 9 activities were determined by using colorimetric assay. (C) The expression of pro- and anti-apoptotic proteins was determined by Western blot analysis. Soluble lysates were matched for protein content and analyzed on Western blot and immunoblots were probed with actin antibody to ensure equal loading. Nuclear translocation of AIF was also determined.

3.3. Ferruginol treatment impairs prostate cancer cell survival

To obtain more insight into the molecular mechanisms mediating ferruginol effects on PC3 cells, the phosphorylation/ expression state of a panel of signal transduction mediators in response to ferruginol was examined. As shown in Fig. 3, PC3 cell treatment with 50 μM ferruginol provoked downregulation of p85 subunit of PI3K and inhibition of AKT. The results presented above indicate that ferruginol should produce a decrease of the survival and anti-apoptotic relevant kinase activities. Accordingly, cells treated with 50 μM ferruginol displayed activation of MAPK p38, an important apoptosis inductor, and a slight inhibition of ERK2. However, the upstream activator of ERK, MEK, was not affected. Apparently, ferruginol impairs prostate cancer proliferation by modulating survival and proliferation signaling cascades.

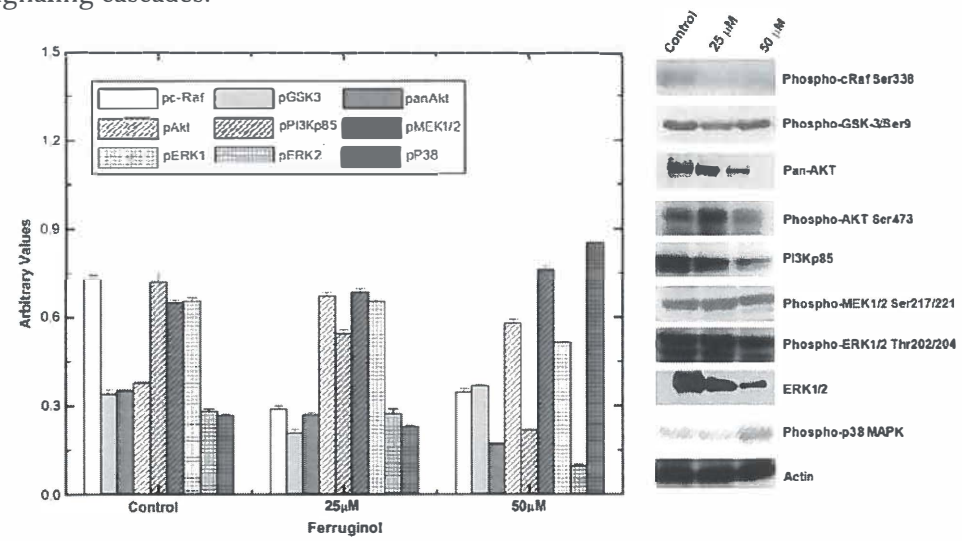


Figure 3. Effect of ferruginol treatment on the function of key mediators involved in the PC3 cells proliferation/survival. Cells were treated with specified concentrations of ferruginol for 24 h and the expression or phosphorylation of the proteins determined by Western blot. Equal loading was confirmed by reprobing them for β-actin.

3.4. Inhibition of cell cycle progression by Ferruginol

By plotting the ratio of the cells in G0 plus G1 against cells in G2/M plus S-phase it is apparent that PC3 cells undergo G0/ G1-phase cell cycle arrest after ferruginol treatment (Fig. 4A). Approximately 2.5-fold of the PC3 population was at phase G0/G1 after exposure to ferruginol for 24 h. We therefore turned to characterize the effect of ferruginol treatment on direct regulators of cell cycle progression. As shown by immunoblot analysis (Fig. 4B), the expression of p21 was increased even at the lowest concentration of ferruginol. On the other hand, the phosphorylated Rb protein and the expression of PCNA were not affected. The level of phosphor-cdc2, the key protein of the cell cycle progression from G2 to M phase, remained unchanged. Ferruginol decreased the level of CDK4, CDK6, cyclin D1 and cyclin D3. This response directly mirrored the ability of ferruginol to induce cell cycle arrest in PC3 cells. We conclude that the combination of reduced mitotic activity and induction of apoptosis accounts for the observed cytotoxic effect of ferruginol on PC3 cells.

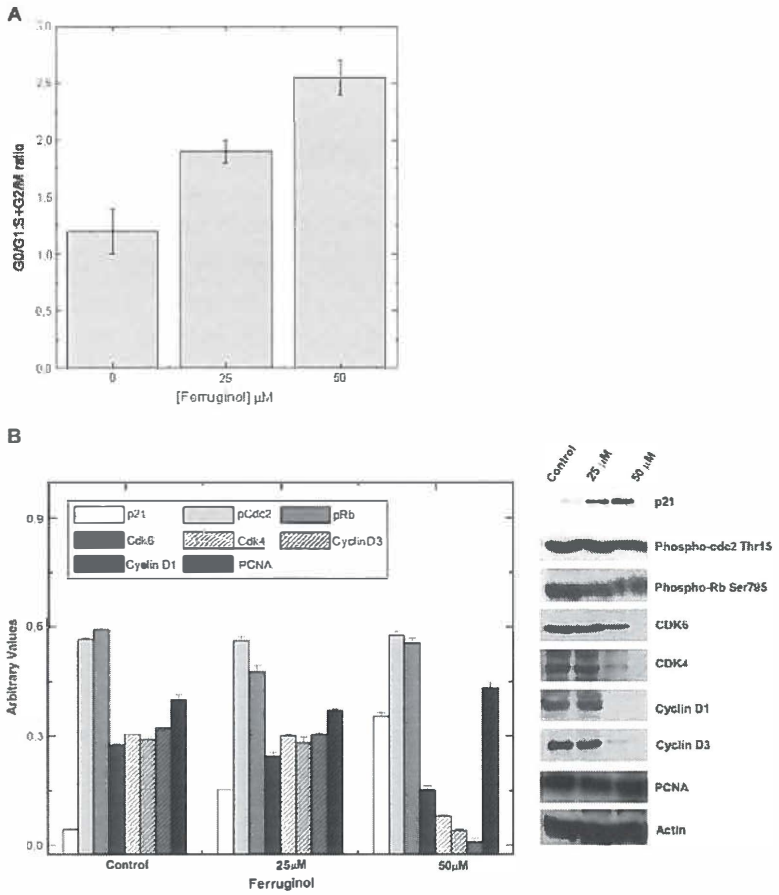


Figure 4. Ferruginol impedes prostate cancer cell proliferation by targeting key cell cycle mediators. After treating PC3 cells with ferruginol for 24 h, cells were harvested, stained with PI and analyzed by flow cytometry (A) or lysed for Western blotting analysis (B). The number of cells in each phase of the profile and ratios of cells in resting phase (G0/G1) versus those undergoing mitosis (S, G2/M) was determined. The expression or phosphorylation of the proteins was determined by Western blot. Equal loading was confirmed by reprobing them for β-actin.

3.5. Ferruginol causes downregulation of IKKa and hypophosphorylation of STATs

The immunoblot analysis data showed that the expression of IKKα was significantly decreased after treatment with ferruginol; however, the total level of NFκB remained unchanged (Fig. 5). In addition, ferruginol caused a

decrease of this transcription factor into the nucleus. The phosphorylation status of STAT 3 and 5, key signaling molecules for many cytokines and growth-factor receptor response, was also evaluated by Western blot. The treatment of PC3 with 50 μM ferruginol was able to decrease the phosphorylation levels of STAT3 (Tyr705) and STAT5 (Tyr694). Interestingly, 50 μM ferruginol caused a strong decline in Hsp27 expression, which can be associated with the increase in the number of apoptotic cells.

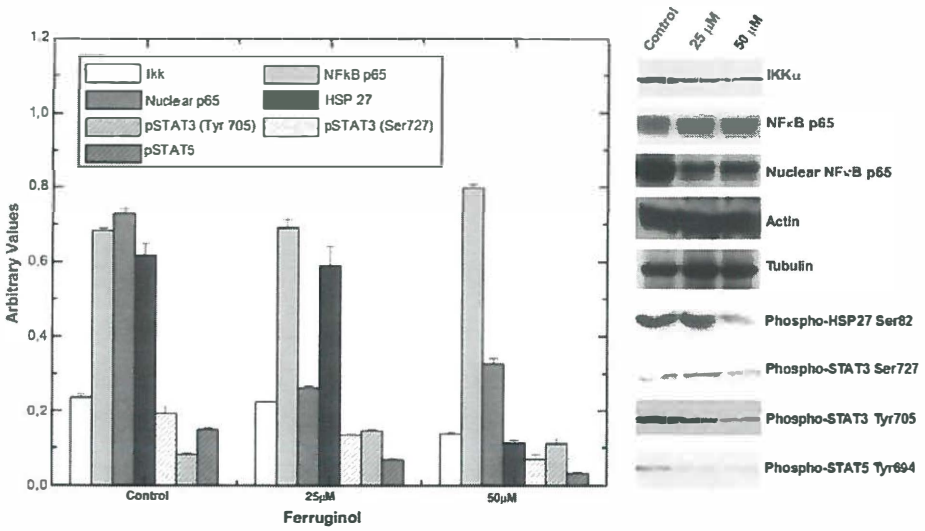


Figure. 5. Ferruginol modulates molecules that play a crucial role in the prostate cancer aggressiveness. Cells were treated with specified concentrations of Ferruginol for 24 h, harvested and total cell lysates were prepared. The expression of IκB kinase, cytosolic and nuclear NFκB, and phosphorylation status of Hsp27 and STAT 3 and 5 were determined by Western blot analysis. Equal loading was probed with actin antibody to ensure equal loading.

3.6. Redox status on PC3 cells treated with ferruginol

Based on the diterpene chemical properties, which can lead to antioxidant and/or oxidant action depending on its concentration, and the importance of reducing equivalents for PC3 cells survival [23], we investigated the effect of ferruginol on PC3 cell GSH metabolism. PC3 cells treated with ferruginol displayed a more oxidizing environment as defined by a decrease of GSH and an increase of GSSG levels (Fig. 6). Both effects were

dose dependent and the highest concentration of ferruginol employed caused a 2-fold change. It is important to note that even at the highest concentration of ferruginol, GSH:GSSG ratio remained almost in equilibrium (GSH:GSSG = 4.4, 2.7 and 1.1 at 0, 25 and 50 μM ferruginol, respectively).

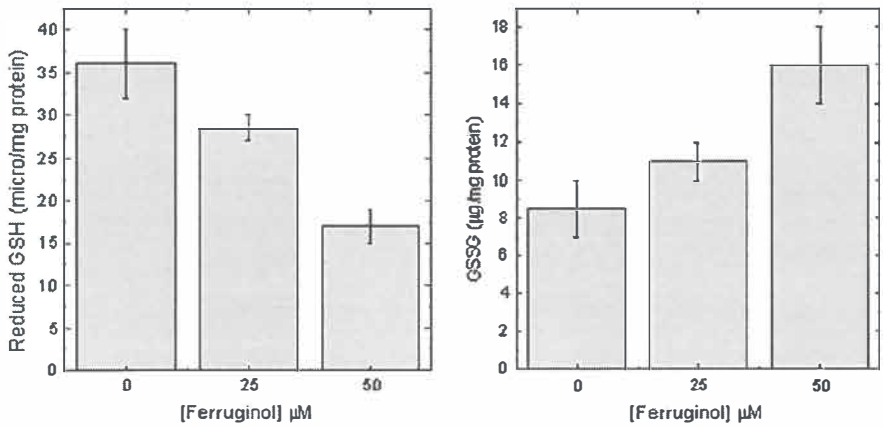


Figure 6. Influence of ferruginol on the level of intracellular GSH and GSSG. Cells were treated with ferruginol for 24 h and the concentration of GSH and GSSG determined as described in Section 2.

3.7. Low molecular weight protein tyrosine phosphatase is modulated by ferruginol

Besides the fact that protein tyrosine phosphatases are highly sensitive to cell redox status, there is some evidence that this class of phosphatases can contribute to tumor cell progression and aggressiveness. Especially LMWPTP has been recognized as a positive regulator of tumor growth [24]. Therefore, to address the possible modulation of LMWPTP by ferruginol, we examined the LMWPTP activity as well as expression. LMWPTP immunoprecipitated from PC3 cells was inhibited around 30% by 50 mM ferruginol (Fig. 7). On the other hand, when the LMWPTP activity was checked after treating the cells for 24 h, this enzyme displayed only 20% of residual activity. Reduced LMWPTP activity is consistent with the change in redox status in response to ferruginol. In addition, treated cells demonstrated downregulation of LMWPTP expression.

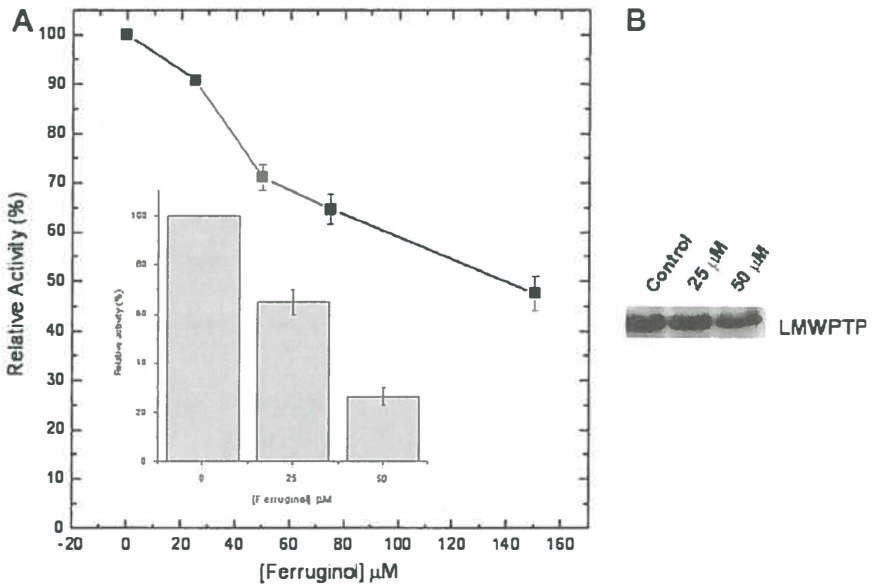


Figure 7. Ferruginol affects low molecular weight protein phosphatase activity and expression. (A) The effect of ferruginol was examined directly in the LMWPTP immunoprecipitated from non-treated PC3 cells and also after PC3 treatment with ferruginol (inset plot). The LMWPTP specific activity was used to determine the relative activity. (B) Expression of LMWPTP was checked by Western blotting.

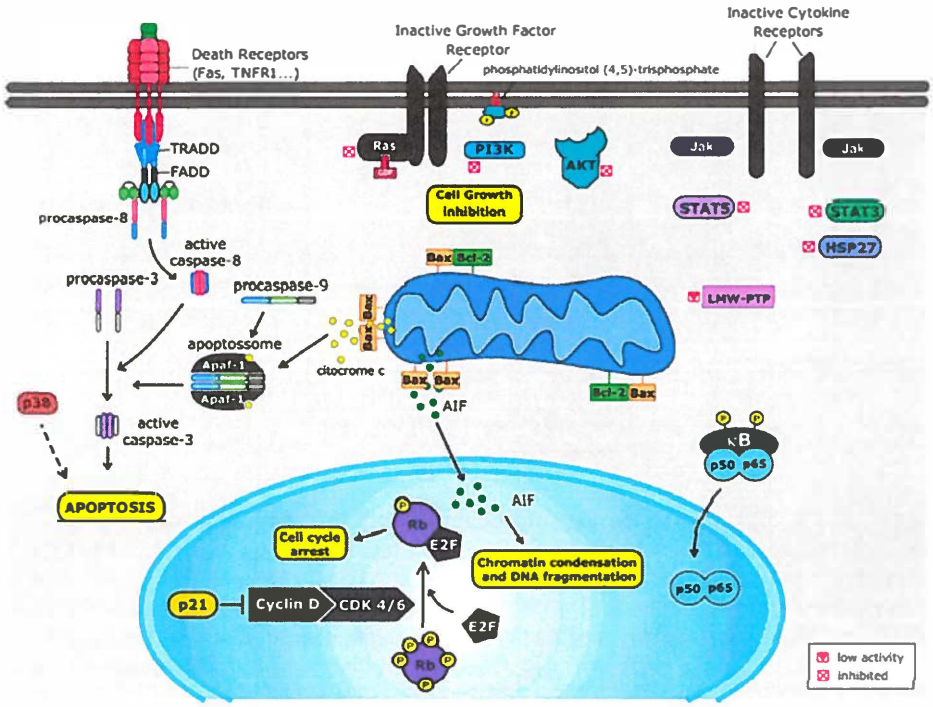


Figure 8. Schematic representation of the molecular mechanism of ferruginol-induced PC3 cell death. Data presented in this report revealed that ferruginol exhibits multi-activities, which culminate with apoptosis induction of prostate cancer. Ferruginol caused inhibition of two important signaling cascade pathways involved in the cell survival/proliferation (Ras/PI3K and Jak/STAT). Additionally treated prostate cancer cells displayed a decrease in the phosphorylated form of Hsp27. Ferruginol- induced apoptosis was accompanied by activation of caspase 3, an increase of AIF expression and maintenance of Bax and Bcl-2 levels. AIF is released from mitochondria and translocated to the nucleus, and participates in peripheral chromatin condensation. In accordance with cell survival diminishing, Ferruginol caused cell cycle arrest. Ferruginol caused an overexpression of protein p21 (a member of the cyclin-dependent kinase inhibitors), and downward expression of cyclin D1, cyclin D3, CDK4 and CDK6, which indicates cell cycle arrest at G0/G1. Importantly, LMWPTP was directly and indirectly modulated by ferruginol, which indicates that this enzyme can be a target for this natural compound.

4. DISCUSSION

Prostate cancer is commonly malignant and it is the second leading cause of cancer-related deaths (after lung cancer) of males in Brazil, with a similar trend in many Western countries (data from Instituto Nacional do Câncer-INCA). Since prostate cancer usually occurs in men aged 50 years and older and because of the increasing life expectancy, its incidence is expected to further rise in the years to come [25]. Chemoprevention and intervention strategies using anticancer agents are considered as promising therapeutic options. The search for new chemopreventive and/or chemotherapeutic agents that are more effective without toxic side-effects has generated great interest in phytochemicals with potential activity in this respect [26]. Suppression of tumorigenesis often involves modulation of signal transduction pathways, leading to alterations in gene expression, cell cycle progression or apoptosis. Apoptosis is considered as an ideal way for destroying damaged cells and also a potential target for chemopreventive elimination of cancer cells [27] and as a consequence targeting signaling elements controlling apoptosis may open novel therapeutic avenues [28,29]. Several plant-derived bioactive agents may have such action, at least as judged from model systems [4,9,30-33]; the present study may add ferruginol to this growing list.

Our results suggest that ferruginol is a negative regulator of cancer cell proliferation. Androgen-independent human prostate cancer cells (PC3 cells) a model that exhibits extreme therapy resistance exhibited, upon treatment with this phytocompound, remarkable induction of apoptosis via extrinsic and intrinsic pathways, as demonstrated by the observation of overexpression of TNFR1 and activation of caspases 8, 9 and 3. The extrinsic pathway for cell death involves plasma membrane death receptors [34]. These receptors trimerize and recruit the adaptor molecule FADD which, in turn, activates caspase 8 and also leads to the activation of downstream execution caspases [35-38]. In both pathways, activation of effector caspases leads to a series of morphological changes that are characteristic for apoptosis [28].

Ferruginol-induced apoptosis and cell growth inhibition were also accompanied by an increase of apoptosis-inducing factor (AIF) expression and maintenance of Bax and Bcl2 levels. AIF was identified as a mitochondrial intermembrane space protein, which is released from mitochondria and translocated to the nucleus, in response to apoptotic stimuli, and participates in peripheral chromatin condensation and the exposure of phosphatidylserine in the outer leaf of the plasma membrane. Increasing evidence supports the

notion that AIF plays an important role in caspase-independent apoptosis [39,40].

Ferruginol, even at lower concentration, caused inhibition of Ras/PI3K cascade and suppression of downstream mitogenic targets such as cyclin D1. Additionally, this diterpene also induced activation of MAPK p38. The phosphatase and tensin homologue (PTEN) gene is deleted in PC3 cells. This phosphatase is defined as a tumor suppressor, since it is the major negative modulator of AKT protein, an important mediator of cell survival. It is therefore important to identify agents that can overcome the therapeutic resistant properties of PTEN deficient tumor cells. Importantly, in accordance with cell survival diminishing, ferruginol caused cell cycle arrest. Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases [41]. Ferruginol caused an overexpression of protein p21 a member of the cyclin-dependent kinase inhibitors, and downward expression of cyclin D1, cyclin D3, CDK4 and CDK6. These data showed the involvement of p21 in ferruginol- induced G1 phase arrest, through binding to and subsequently inhibiting the cyclin-CDK activity. The active complex of cyclin D/CDK4 targets the Rb protein for phosphorylation, allowing the release of E2F transcription factors that activate G1/S-phase gene expression. Importantly cdc-2, a key protein responsible for the entry of the cell from G2 to M phase, remained unchanged. Cell cycle regulation and its modulation by various plant-derived agents are gaining widespread attention in recent years. A large number of phytochemicals has been shown to inhibit cell cycle progression of various cancer cells [42].

Ferruginol decreased the phosphorylation level of STAT3, STAT5 and Hsp27. STATs are latent cytoplasmic transcription factors consisting of seven mammalian members. They become phosphorylated on Tyr residues upon activation, a post-translational modification that is critical for dimerization, nuclear import, DNA binding, and transcriptional activation [43]. The activation of STATs is mediated by the action of an upstream Janus kinase (JAK), usually JAK1 or JAK2, showing that the JAK cascade might itself be a target for therapy in prostate cancer. Ahohen and coworkers [44] demonstrated that STAT5 is activated in a significant number of human prostate cancer specimens. Additionally, these authors also reported induction of apoptosis via caspases 9 and 3 activation dependent on inhibition of STAT5 phosphorylation. Activated STAT3 was reported in many types of malignancies, such as myeloma, head and neck cancer, breast cancer, and prostate cancer [45]. Recently, it has been demonstrated that inhibition of STAT3 in tumors impeded vascular endothelial growth factor production

[46]. Data from the literature have identified Hsp27 as a modulator of STAT3-regulated apoptosis after androgen ablation. Hsp27 is a 27 kDa protein of which expression is seen to be correlated with an increase of survival in response to a wide variety of physiological and environmental insults including heat, reactive oxygen species and anticancer drugs. Indeed analysis by co-immunoprecipitation and immunofluorescence confirmed that Hsp27 is able to interact with STAT3 and that STAT3 levels correlate directly with Hsp27 levels. There are some reports in the literature demonstrating that the prostate cancer Hsp27 level increases after androgen ablation and that this protein is highly expressed in androgen-independent tumors, and inhibition of Hsp27 in prostate cancer cells can increase the number of apoptotic cells (G0/G1), an event that seems to be associated with the decrease in the STAT3 levels [47]. These findings indicate that the anti-apoptotic effects of Hsp27 are associated with its ability to interact and stabilize the STAT3 molecule, leading to more resistant prostate cancer cells. In accordance with this notion, our results show a decrease in the phosphorylated forms of Hsp27 and STAT3, when PC3 cells were treated with ferruginol at the concentration of 50 mM, suggesting that the pro-apoptotic and anti-proliferative actions of ferruginol might be associated with diminished function of STAT3 through the decrease of Hsp27 levels.

Another important finding is that ferruginol did not induce the NFκB translocation to the nucleus; this result is in agreement with the findings of Rodriguez and coworkers [17] related to the anti-inflammatory effect of ferruginol.

Recently, Chaiswing and collaborators [23] reported in a very well designed paper the effect of cellular redox state on prostate cancer cell growth in vitro. These authors demonstrated that during PC3 cells growth, these cells require higher ratio of reduced glutathione (GSH)/glutathione disulfide (GSSG). Based on this observation, we evaluated the redox status toward PC3 cell treatment with ferruginol. This diterpene caused a decrease of GSH and increase of GSSG, indicating a dominant effect in favor of oxidizing equivalent. Several signaling mediators can be modulated by redox modifiers, including protein tyrosine phosphatases [48, 9]. To investigate the effect of ferruginol on PTPs we chose LMWPTP. The rational reason for this was based on the following aspects: (a) we have observed a high level of this phosphatase in PC3 cells and (b) Chiarugi and collaborators [24] have reported the importance of this enzyme for cancer cell growth. Interestingly, we observed a direct effect of ferruginol on LMWPTP but also there was a correlation between cellular oxidizing equivalents and inhibition of this enzyme. The oxidation of catalytic

site cysteine of PTPs, such as LMWPTP, leads to the transformation of the sulfhydrylic residue in sulfenic acid and the consequent inactivation of the enzyme due to its inability to form cysteinyl-phosphate intermediate during the first step of the catalysis [48]. Altogether, our results demonstrate that ferruginol can act as a chemical and genetic modulator of LMWPTP. These data indicate that at least in part, the anti-proliferative ction of ferruginol is dependent on changing cellular redox, which is in agreement with the protective effect of GSH. This observation also confirms the importance of reducing equivalents for PC3 cell survival, as recently reported [23]. Further experiments to clarify the role of LMWPTP on prostate cancer progression are currently in progress in our laboratory.

5. CONCLUSION

One of the challenges of cancer therapy is to combine efficacy with few side effects and consequently improve the quality of life of the patient. Prostate cancer represents a spectrum of diseases in which the cost of cure may be substantial, with short- and long-term side effects. Therefore, new agents are needed to extend survival, improve cure rates, and avoid undesired treatment-related toxicities. In this scenario, there are at least two aims: (a) to provide therapeutic agents with a very specific target and (b) to discover agents which present differential action mechanisms in comparison with the traditional chemotherapy. In this context, ferruginol appears as an interesting bioactive compound, since it exhibits multi-activities in the signal transduction/biochemical aspects in prostate cancer cells. In summary, ferruginol negatively modulates signaling cascades, which are known to be defective in some types of prostate cancers, namely Ras/PI3K and Jak/STAT, as well as cell cycle regulators (Fig. 8). Importantly, we demonstrated for the first time that LMWPTP is directly and indirectly modulated by ferruginol, which indicates that this enzyme can be a target for this natural compound. Besides affecting signal transduction triggered by TNFR1, ferruginol also affected mitochondria permeability as demonstrated by the presence of nuclear AIF (protein involved with chromatin condensation and DNA fragmentation); however, the Bax:Bcl2 ratio remained unchanged. In general, this study provides an overview of biochemical aspects which were affected by ferruginol and in turn confirms its anti-tumor activity. This type of investigation can contribute to the development of "smart" drugs.

ACKNOWLEDGMENTS

Financial support by FONDECYT (Grant No. 1060841) and the Programa de Productos Bioactivos, University of Talca is gratefully acknowledged. C.A. thanks the Universidad de Talca for a doctoral grant. The authors acknowledge financial support by the Brazilian Agencies: Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundo de Apoio ao Ensino, à Pesquisa e à Extensão.

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Chapter 7

Summarizing Discussion

Future Perspectives

Nederlandse samenvatting

Acknowledgements

1. SUMMARIZING DISCUSSION

Although cancer chemotherapy has been one of the major medical advances in the last few decades, cancer remains a very serious health problem worldwide. Considering the different causes, the different tissues affected, the different symptoms and the different response to treatment, cancer is a very complex and still incurable disease. Much effort has been directed at comprehending carcinogenesis and a lot of progress has been achieved. However, there is still no effective treatment for most cancers, and a new challenge arises when some patients, for no apparent reason, develop resistance to the treatment. In this *status quo*, studies about molecular mechanisms of carcinogenesis, chemoresistance acquisition and apoptosis induction of cancer cells can contribute enormously to overcome the cancer challenges. With the studies described in this thesis we strive to contribute to the ongoing battle against cancer. In this last section of the thesis, we summarize the work done and discuss the implication of the studies.

In **Chapter 1**, we invite the reader to understand the structure of this thesis, showing the importance of studying the molecular mechanism and effects of natural compounds as chemotherapeutics and giving some successful examples of these kinds of studies in cancer treatment.

In **Chapter 2** we summarize the potential role of phosphatases in the treatment of hematological malignancies. This article describes extensively the protein phosphatases implicated in three major kinases pathways deregulated in hematological malignancies, the JAK-STAT signaling, Ras–Raf–MEK–ERK signaling and PI3K–PKB signaling. Many papers have discussed the role of kinases in cancer progression but relatively few have given attention to phosphatases. It is important to remember that the regulation of phosphorylation levels which control activation and deactivation of proteins is very delicate and controlled by both kinases and phosphatases. Therefore, we feel it would be a gross negligence to ignore the role of phosphatases in carcinogenesis. Our literature studies suggest that there may be a potential relevance for phosphatases in cancer treatment, and we hope to prompt an increase in the interest for these enzymes in cancer studies.

In the **Chapter 3,** the role of Hedgehog (Hh) signaling in chemoresistance acquisition is studied in myeloid leukemia cells. Multidrug Resistance (MDR) against chemotherapy remains one of the major challenges in overcoming leukemic diseases. Thus the characterization of signaling pathways sustaining MDR is fundamental for success in therapies for MDR leukemias. In this study, we show that chemoresistant myeloid leukemia cells

display activation of Hh signaling. Inhibition of this pathway reverses chemoresistance in Lucena-1 cells. The effect of Hh inhibitors is probably dependent on their ability to decrease P-gp expression. This explains why Hh inhibitors can sensitize Lucena-1 cells but not K562 cells, which do not express P-gp, or GlC4-doxo cells which express MRP-1, but not P-gp. Interestingly, overexpression of constitutively active Smoothened, a downstream target of Hh, protects K562 leukemia cells from vincristine-induced cell death. These data provide evidence that the Hh pathway is not only sufficient but also essential for resistance to classical chemotherapy in myeloid leukemia. Furthermore, this study can be relevant for early detection of resistance, for example the expression of Ptch-1 may be a marker in patients developing chemoresistance.

In **Chapter 4** we studied the cytotoxic effect of the flavonoid fisetin in myeloid leukemia cells. Fisetin can induce cell death in HL60 leukemia cells but not in peripheral human lymphocytes. After publication of our paper, numerous other studies regarding the effect of fisetin in cancer cells have come out. Confirming our result that fisetin displays antiproliferative effects only in tumor cells, Khan and co-workers showed a cytotoxic effect in prostate cancer cell lines but not in epithelial prostate cells [1]. The molecular mechanism of the cytotoxic effect induced by fisetin in tumor cells has received vast attention [2-5]. The antiproliferative activity of fisetin was shown to be dependent on the activation of death receptor- and mitochondrial-dependent pathways and subsequent activation of caspase cascades [3]. Nevertheless, not only the classical apoptosis pathway as shown in LNCap prostate can be activated by fisetin, [1], but also autophagic-programmed cell death may occur, as was the case in PC3 prostate cancer cells [5].

Fisetin anticarninogenic effect, antioxidant and anti-inflamatory activity are clearly defined via inhibition of cell proliferation and free radical scavenging respectively [6]. However, fisetin can also be a pro-oxidative agent in cancer cells, thereby inducing cell death. In Chapter 4 we observed this pro-oxidative effect of fisetin. We showed inactivation of aconitase, a sensitive mitochondrial marker for oxidative stress, as a result of fisetin treatment. Reactive oxygen species (ROS) can induce cell death through activation of TNF receptor and induction of JNK and p38 signaling [7], which was exactly what we observed in HL60 cells treated with fisetin. In addition, fisetin decreases levels of ERK phosphorylation, which is essential for its kinase activity. Other studies have also described fisetin-induced inhibition of kinase activity involved in proliferation signaling pathways [2-5]. For instance, treatment of prostate cancer cells with fisetin inhibits mTOR and

AKT activities [5], and in human lung cancer cells, fisetin can induce inhibition of ERK, leading to inhibition of cell adhesion, migration and invasion in addition to cell death [2], showing an anti-metastatic effect of flavonoid.

Another interesting observation we made in Chapter 4 was that NFκB levels were reduced in HL60 cells upon treatment with fisetin. NFκB is a transcription factor involved in the production of cytokines acting as a pro-inflammatory modulator. This finding indicates the anti-inflammatory property of fisetin. Recent studies have shown a similar effect of fisetin in human prostate cancer cells and human colon cancer cells, where fisetin significantly decreased the nuclear levels of NF-κB in a concentration-dependent manner [2] [4]. This effect could be correlated to the anti-inflammatory effect of fisetin observed through inhibition of COX-2, but not COX-1, or prostaglandin E production [4]. In conclusion, we and others have shown that fisetin may hold promise as a chemotherapeutic agent in diverse tumor types.

In **Chapter 5**, the effect of apigenin, a flavonoid common in human diet, was investigated in leukemia cell lines. Studies into the apigenin effects on cancer cells may contribute to the understanding of its possible chemopreventive and chemotherapeutic properties. In this chapter we show that apigenin induces cell-cycle arrest in G2-M in HL60 cells whereas TF-1 cells were arrested in G0/G1 phase. Some research groups have shown that apigenin can induce cell cycle arrest in different phases, depending on the cell type [8] [9]. Numerous studies have correlated G2/M arrest with apoptosis induction, as was the case in our study. On the other hand, G0/G1 arrest is observed on reduction of mTOR activity [10] and has been associated to autophagy [11], evocative of TF-1 cells in his study. Interestingly, apigenin down regulates mTOR in TF1 cells without affecting PTEN, showing that its effect is either independent or downstream of this lipid phosphatase which normally plays a regulatory role in mTOR pathway activation. Hence apigenin might be a very attractive therapeutic agent for treating tumors carrying inactivated PTEN. Apigenin-induced autophagy in TF-1 cells occurs via inactivation of p70S6K, an inhibitor of autophagy. Unlike TNF-α-induced autophagy, apigenin does not increase the level of sentinel autophagy proteins.

Although the final consequence of apigenin treatment is different in HL60 and TF-1, they share one common target: the Src/JAK/STAT pathway, a major mediator of cytokine activity. Our results suggest that JAK/STAT signaling pathway is a principal target of apigenin. As the JAK/STAT pathway is one of the most deregulated pathways in not only hematological

malignancies (as reviewed in Chapter 2), but other tumors as well [12], a broader application of apigenin treatment of cancer may be envisioned.

An interesting note to be made is that whereas apigenin affects PKB activity in HL60 cells, it does not influence this molecule in TF1 cells. This correlates with the finding that PTEN activity is increased in the former cell line, but not in the latter. Conversely, a more pronounced decrease of JAK/STAT3/5 pathway activity was observed in TF1 cells as compared to HL60, which correlated with the finding that apigenin causes LMWPTP activation in TF1 but not HL60 cells. These data show that the antiproliferative effect of apigenin on leukemic cells is as much attributable to its effects on phosphatases as its effects on kinases, thus strengthening the notion that the study of phosphatases in oncogenetic processes and treatment are very important, and deserve more attention.

One of the most important findings in this study was that apigenin impairs the cytotoxic effect of a chemotherapeutic agent that acts on M phase progression. Causing a cell cycle arrest in G0/G1 phase in TF1 cells, apigenin hampers the cell death induced by vincristine, a clinical chemotherapeutic which binds to tubulin dimers, inhibits microtubule formation and arrests mitosis in metaphase. Interestingly, apigenin did not impair the effect of chemotherapeutics with targets other than the M phase, such as mitoxantrone (which inhibits S-phase) and Imatinib (which inhibits mainly ABL kinase and c-KIT), implying that its action might be specific for M-phase-affecting chemotherapeutics.

This study concludes that apigenin is a potential chemopreventive and chemotherapeutic agent in leukemia, but the consumption of apigenin must be carefully studied in patients treated with anti-mitotic chemotherapy.

In **Chapter 6**, ferruginol, a natural compound found in Chilean trees (Podocarpaceae) was studied in prostate cancer cells. This diterpene negatively modulates cancer cell proliferation and positively regulates apoptosis in PC3 cells, a model of very aggressive prostate cancer. Both intrinsic and extrinsic apoptosis pathways were induced by ferruginol in prostate cancer cells. A high ratio of Bax:Bcl2, high nuclear AIF levels and caspase 9 activity are the modifications observed in the intrinsic apoptosis pathways. High expression of TNFR1 and high activity of caspase 8 were also observed in ferruginol-treated cells, indicating the activation of the extrinsic apoptosis pathway. Consequently, ferruginol increases caspase 3 activity, where these two pathways converge. In addition to its effect on apoptosis induction, ferruginol is able to inhibit proliferation and survival signaling pathways such as the PI3k/AKT and MEK/ERK pathways. Cell-cycle arrest in G0/G1 phase was

observed, corroborating with high expression of p21 protein and low levels of CDK6, CDK4, cyclin D1 and cyclin D3. One of the most interesting observations in this study was the inhibition of Hsp27 by ferruginol. Inhibition of Hsp27 destabilizes and inhibits STAT3, and can thereby reduce cell survival.

As shown by others, diterpenes possess anti-inflammatory properties and in our model, we observed less translocation of NFκB into the nucleus in ferruginol treated cells. Furthermore, the oxidative state of the prostate cancer cells was altered upon ferruginol treatment, and obtaining higher GSH/GSSH ratios may be the initial cause of cell death induction in PC3 cells.

Although the natural compounds studied in this thesis display different mechanisms leading to the same final effect, i.e. cell death and cell cycle arrest, they also share similarities in their modes of action. Ferruginol and apigenin can induce apoptosis via caspase-dependent mechanisms, as was observed for prostate cancer cells treated with ferruginol and HL60 cells with apigenin. In both these studies, PI3K/AKT and JAK/STAT signaling were inhibited and MAPK p38 activated by the natural compounds.

Fisetin also induces caspase-dependent apoptosis [3] and activates MAPK p38. Both ferruginol and fisetin were able to induce oxidative stress, and although not studied by us, apigenin has been shown to induce apoptosis through ROS production [13]. ROS can act as a cell death inducer by causing damage in DNA, lipid peroxidation and protein oxidation, in addition to playing a role in cell signaling. ROS activates signaling cascades that trigger gene expression, through directly oxidizing components of signaling pathways and interfering in their activity, ultimately changing gene expression by modifying the activity of transcription factors [14]. MAP kinase signaling has also been show to be target of ROS [15].

One class of enzymes that can be inactivated by ROS is that of the protein tyrosine phosphatases. Protein tyrosine phosphatases (PTPs) share a common CX2R active site motif and an identical catalytic mechanism based on the participation of a crucial cysteine residue [16]. When this cysteine is oxidized, PTP activity is inhibited. One of possible mechanisms through which fisetin could increase protein phosphorylation levels is through the inhibition of phosphatases by ROS. Indeed, we observed a drastically reduced activity of the LMWPTP by ferruginol treatment of PC3 cells and apigenin treatment of HL60 cells. In the case of TF1 cells, the ROS production might be not related to the apigenin effect, since LMWPTP was activated by apigenin treatment. LMWPTP has been shown to be up-regulated in different human cancers and it can be correlated with bad prognosis [16].

TF1 cells displayed a completely different cell death mechanism in response to apigenin treatment. These studies can be considered as a model for apoptosis resistance. In this case, apigenin was not able to induce apoptosis, but instead autophagy was observed. A very interesting finding was the increased LMWPTP activity upon treatment of TF1 cells with apigenin. As described in Chapter 2, the role of LMWPTP in hematological malignancies is not well known, and nothing was yet described about the role of LMWPTP in cancer resistance. We now suggest a link between resistance and LMWPTP because apigenin treatment increases LMWPTP activity in a dose-dependent fashion. As apigenin increases LMWPTP activity, and impairs vincristine induced cell death, LMWPTP might be the responsible for the failure of apigenin to induce cell death in TF1 cells. Hence we emphasize the importance of studying phosphatases for understanding the success or failure of a treatment.

The mechanism by which the natural compounds are taken up by the cells may contribute to their biological effect. For example, the antioxidant and antibacterial properties of flavonoids are related to their interaction with biological membranes [17, 18]. Moreover, the organization of the lipids in the membranes is essential for signal transduction, especially for tyrosine kinases signaling, which requires adaptors, scaffolds and enzymes. It is established that activation of some tyrosine kinase receptors involves lipid rafts, the cholesterol and sphingolipids enriched microdomains in the plasma membrane [19]. Some examples of these include T-cell [20] , B cell [21], Ephrin B1 [22], Hedgehog receptors [23]. As all flavonoids intercalate into phosphatidylcholine bilayers [24]. phosphatidylcholine is present in a non-lipid raft area, suggesting that flavonoids might interfere in rafts formation and thus, block tyrosine kinases receptor activation. However, their effect on membrane proteins is not restricted to their effect on membranes per se. Flavonoids can also interact directly with proteins in the membrane, affecting their activity. Flavones apigenin and acacetin and flavonols morin and myrecetin are effective inhibitors of MRP1. Apigenin was demonstrated to stimulate ATPase activity in drug-resistant small cell lung cancer [25] and binds to P-gp [26] and MRP1 [27]. Intriguingly, depending on the substrate, apigenin can inhibit or stimulate MRP1 activity: apigenin decreases accumulation of daunomycin while increasing the accumulation of vinblastin in Panc-1 cells [28]. Fisetin decreases the efflux of both substrates, indicating the stimulation of MRP1 activity [28]. Additionally to the effects on membrane lipids and proteins, flavonoids can pass through the plasma membrane and reach the cytosol, where other interactions can take place. Although flavonoids can be detected within the cells minutes after treatments,

they diffuse very slowly across lipid membranes or not at all.. Therefore, a transport system is required to achieve the rapid flavonoid uptake [29]. Schramm and coauthors showed that the uptake of the flavonoid morin by epithelial cells is increased by the presence of ATP in culture medium and using MRP1 inhibitors the uptake is reduced [29]. Krotta and coauthor, using an oocyte model, showed that Na+/glucose transporter is not responsible for apigenin uptake. Despite some studies have shown how morin enters in the cells, other flavonoids do not necessary has the same pattern. Changes in number and position of the hydroxyls groups can change the way how flavonoids are taken up by the cells. Thus, the uptake of apigenin and fisetin remains to be solved.

Together this thesis investigates the molecular mechanisms mediated by natural compounds and the molecular mechanism in resistance acquisition.

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2. FUTURE PERSPECTIVES

As mentioned above, our literature and experimental studies suggest that there might be a bigger role for phosphatases in hematological malignancies and other cancers then so far assumed. Although we found in all our studies that natural compounds affect kinases, our results in Chapter 5 suggest that phosphatase activities are also modulated. We have already established a role for PTEN and LMWPTP in apigenin-induced cell death in leukemic cells and modulation of LMWPTP activity by ferruginol. It is conceivable that other phosphatases are also regulated by ferruginol, apigenin and fisetin. As our studies suggest a significant impact of natural compounds on cell cycle regulation, it would be of interest to study their effect on cell cycle division phosphatases such as CDC25. Indeed, it has recently been shown that the natural compound indole 3-carbinol initiates breast cancer G1 arrest by modulation of CDC25 expression [30]. In addition, protein phosphatase 2C mediates caspase dependent apoptosis in HL60 cells induced by the diterpene pisiferdiol [31], further demonstrating the importance of studying phosphatases in natural compound mediated cell killing.

Studies into the mechanism of how ferruginol, apigenin and fisetin are taken up by the cells are relevant to find to specific targets. Furthermore, natural compounds can be a platform for synthesis of new drugs. Modification of the molecular structure can improve the desired biological effects and produce fewer side effects. In addition, although many natural compounds have already been shown to be tolerated in humans, clinical implementation of these compounds may be hampered by their differential interaction with, and effect on, signaling and cellular function of other agents, as was shown by our finding of the negative effect of apigenin on chemotherapy. Careful further studies with hedgehog inhibitors, fisetin, apigenin and ferruginol are obviously necessary, and validation of the effects of new drugs in animal models is essential for a safe future use in humans.

Nevertheless, the idea of going back to nature for treatment of diseases is an appealing one, and, according to our data, one that shows great promise.

3. NEDERLANDSE SAMENVATTING

Kanker is een van de meest voorkomende ziektebeelden in Nederland. In 2008 werden in totaal 89200 nieuwe gevallen geconstateerd, waarbij borst (14%), long (12%), prostaat (11%), colon (9%) en hematologische aandoeningen (8%) het meest prevalent waren (Vereniging integrale kankercentra, http://www.ikcnet.nl). Ondanks voortschrijdend medisch inzicht betreffende de behandeling van verschillende vormen van kanker er sterven momenteel in Nederland evenveel mensen aan kanker als aan hart en vaatziekten. (Nationaal Kompas Volksgezondheid, RIVM). Naast het feit dat voor sommige vormen van kanker geen adequate bestrijdings mogelijkheden beschikbaar zijn, gebeurt het regelmatig dat tumor cellen resistent worden tegen de gegeven chemotherapieën. Ook komt het in sommige gevallen voor dat na volledige verdwijning van de tumor een recidief optreedt, die vaak moeilijker te bestrijden is dan de originele ziekte. Er is derhalve een dringende behoefte aan ontwikkeling van nieuwe therapeutische kankerbestrijding mogelijkheden.

Alle celfuncties worden in grote mate bepaald door signaal transductie routes, bestaande uit intracellulaire enzymen die worden aangezet door extracellulaire stimuli, waaronder groeifactoren. Wanneer er in deze signalering cascades fouten optreden is het mogelijk dat de cel te heftig reageert op omgevingsfactoren, of afwijkend gedrag vertoont. In veel kankervormen zijn verstoringen in signaaltransductie routes aangetoond, die leiden tot de voor kanker karakteristieke ongeremde celdeling en celoverleving. Het is dan ook niet verwonderlijk dat veel onderzoek is gericht op het ontwikkelen van medicijnen die aangrijpen op deze verstoorde signalering. Recentelijk is gebleken dat sommige stoffen uit natuurlijke producten zoals groente, fruit, bomen en planten, in staat zijn cellen te beïnvloeden door te interfereren in deze signaaltransductie routes, en de mogelijke toepasbaarheid van deze in de natuur voorkomende stoffen in de bestrijding van verschillende ziektebeelden krijgt dan ook steeds meer aandacht.

Met het werk beschreven in dit proefschrift proberen wij een bijdrage te leveren in de strijd tegen kanker. Hierbij hebben wij ons voornamelijk gericht op het effect van enkele natuurlijke stoffen op de groei van bloed en prostaat kanker, en de signalerings eiwitten die daarbij betrokken zijn.

In **Hoofdstuk 1** wordt de opzet van dit proefschrift uitgelegd. Het belang van de studie naar het effect van natuurlijke stoffen als kankerremmende middelen en de moleculaire mechanismen daarvan wordt besproken. Enkele studies uit de literatuur, die een succesvolle remming van kanker celgroei door natuurlijke stoffen beschrijven, worden aangehaald om het belang van natuurlijke kankerremmende stoffen als mogelijke nieuwe chemotherapeutica te illustreren.

In **Hoofdstuk 2** onderzochten wij aan de hand van literatuurstudie de mogelijke rol van phosphatases in de behandeling van hematologische aandoeningen. Een belangrijke manier van het doorgeven van intracellulaire signalen is de fosforylering van eiwitten en lipiden. Enzymen die in staat zijn om een fosfaatgroep aan een eiwit/lipide te bevestigen, de zogenaamde kinases, zijn veelvuldig bestudeerd. Met name hun rol in de ontwikkeling van kanker heeft veel aandacht gekregen. Drie belangrijke kinasengereguleerde signalering cascades, waarvan een verstoring is aangetoond bij bloed kankers, worden in Hoofdstuk 2 beschreven; de JAK/STAT, Ras-Raf-MEK-ERK en de PI3K-PKB routes.

Naast het aanzetten van signalerings routes, veelal door kinasen, moeten deze cascades ook weer worden uit gezet. Fosfatasen zijn in staat fosfaatgroepen van eiwitten en lipiden te verwijderen, en worden daarmee veelal gezien als negatief regulerende factoren in signaal transductie. Hun rol in kankercel biologie wordt daarmee echter onderschat, een verhoogde activiteit van signalen zou theoretisch immers kunnen worden verklaard door zowel een verhoogde activiteit van kinases, als een verlaagde activiteit van fosfatasen. Bovendien blijkt uit literatuur studie dat sommige fosfatasen, waaronder CD45, SHP-2 en SHIP, naast een inactiverende functie ook een activerende rol kunnen uitoefenen. In Hoofdstuk 2 beschrijven wij hoe deze en andere fosfatasen betrokken lijken te zijn bij de pathogenese van hematologische kankers, en mogelijk een aangrijpingspunt zouden kunnen vormen voor de behandeling van deze ziektebeelden.

In **Hoofdstuk 3** gaan we dieper in op de ontwikkeling van resistentie tegen chemotherapeutica door tumor cellen. Het overkomen van deze zogenoemde multidrug resistentie (MDR) blijft een van de grootste uitdagingen in de bestrijding van kanker. Een van de mechanismen waarmee tumor cellen resistentie verkrijgen is door de expressie van membraaneiwitten waarmee antikankermiddelen uit de cel worden gepompt. Wij beschrijven hier de rol van Hedgehog (Hh) signaaltransductie in de expressie van een van deze pompen, P-gp. Lucena-1 leukemische cellen hebben een hogere mate van activatie van de Hh signalerings route dan hun zuster cellijn K652, wat gepaard gaat met een verhoogde resistentie tegen chemotherapeutica. Geforceerde expressie in K562 cellen van constitutief actief Smoothened, een van de eiwitten in de Hh signaleringsroute, maakt ook deze cellen resistentie tegen antikankermiddelen. Inhibitoren van de Hh signaaltransductie maken Lucena-1 cellen weer gevoelig voor chemotherapeutica, wat waarschijnlijk veroorzaakt wordt doordat inhibitie van Hh activiteit de expressie van P-gp verlaagd. Dit verklaart ook waarom K562 en GLC4-doxo, die geen P-gp maar andere multidrug pompen tot expressie brengen, geen invloed ondervinden van Hh inhibitoren.

De studies beschreven in dit hoofdstuk lichten geven een mogelijk mechanisme waarop tumoren ontsnappen aan de celdodende eigenschappen van chemotherapeutica, namelijk het opreguleren van Hh signalering en P-gp expressie. Het onderzoeken van Hh activiteit bij leukemie patiënten zou mogelijk een voorspellende waarde kunnen hebben voor het al dan niet van nut zijn van Hh inhibitoren in de behandeling van resistente tumoren.

In **Hoofdstuk 4** bestuderen we het celdodende effect van de natuurlijke stof fisetin in myeloide leukemie. De flavonoide Fisetin, een stof die van nature voorkomt in groente en fruit, kan celdood induceren in de leukemische cel lijn HL60, maar niet in normale witte bloedcellen uit het perifere bloed. We tonen aan dat fisetin leidt tot activatie van de moleculair stress signalen p38 MAPK en JNK, terwijl activatie van het celoverleving signaal ERK1/2 juist verminderd wordt. Ook de expressie van de transcriptie factor NFκB, waarvan eerder een rol in celdood werd aangetoond, wordt verhoogd door fisetin behandeling van HL60 cellen. In totaliteit werd er in fisetin-behandelde cellen een verhoogde mate van eiwit fosforylering gevonden, wat hoogstwaarschijnlijk een gevolg was van een verminderde fosfatase activiteit in deze cellen. Tevens toonden we aan dat fisetin in HL60 cellen een verminderde activiteit van aconitase, catalase en superoxide dismutase bewerkstelligde. Deze enzymen zijn allen betrokken bij de afbraak van zuurstofradicalen; een verlaging van deze enzymactiviteit resulteert derhalve in een verhoogde hoeveelheid zuurstofradicalen in de cel. Zuurstofradicalen zijn toxisch voor cellen en kunnen via activatie van p38 en JNK leiden tot celdood. De studies beschreven in dit hoofdstuk laten zien dat de natuurlijke stof fisetin in staat is leukemische celfgroei te remmen door de afbraak van zuurstofradicalen te remmen en daarmee signaaltransductie routes aan te zetten die leiden tot celdood.

Een andere flavonoide met antitumor activiteit, apigenin, werd bestudeerd in **Hoofdstuk 5.** Allereerst toonden we aan dat deze stof, die veel voorkomt in onder andere appels en thee, de groei van leukemische cel lijnen HL60 en TF-1 remt. HL60 cellen waren echter gevoeliger voor behandeling met deze flavonoide. Analyse van de moleculaire mechanismen betrokken bij de apigenin-geinduceerde remming van celgroei toonden aan dat de twee leukemische cel lijnen op verschillende manieren reageerden op deze stof. HL60 cellen werden in hun celdeling geblokkeerd in de G2/M fase, wat resulteerde in een geprogrammeerde celdood (apoptosis). Dit was waarschijnlijk het gevolg van een remming van de eiwitten PKB, STAT3, STAT5 en Src, allen nodig voor de overleving van cellen. In TF-1 cellen werd ook remming van STAT3, STAT5 en Src, maar geen remming van PKB. In deze cel lijn werd echter wel inactivatie van de enzymen mTOR en S6 gevonden, die geassocieerd zijn met een andere vorm van celdood; autofagie. Autofagie is een proces waarbij een cel in tijden van stress zichzelf 'opeet' om

energie te sparen en zodoende langer in leven te kunnen blijven, en wordt gekenmerkt door de aanwezigheid van intracellulaire dubbel membraan vacuoles die cytoplasmatisch materiaal bevatten. Als de stress lang genoeg aanhoud zal de cel uiteindelijk doodgaan, bij verdwijnen van de stress kan de cel zich herstellen. Met behulp van electronen microscopie werd de inductie van dubbel membraan vacuoles door apigenin in TF-1 cellen inderdaad aangetoond, wat gepaard ging met een blokkade van de cel cyclus in G0/G1 fase. Een belangrijke bevinding hierbij was tevens dat apigenin de werking van sommige chemotherapeutica op TF-1 cellen remde, wat uit het oogpunt van kankerbestrijding uiteraard onwenselijk is. Men zal dan ook uiterst voorzichtig te werk dienen te gaan indien natuurlijke stoffen als apigenin in de kliniek een gebruik zullen vinden. Een interessante bevinding in deze studie is dat de verlaagde fosforylering van PKB in HL60 cellen gepaard gaat met een verhoogde activiteit van de fosfatase PTEN, terwijl in TF-1 cellen de verlaagde fosforylering van STAT3/5 gepaard gaat met een verhoogde activiteit van de fosfatase LMWPTP. Deze data tonen aan dat fosfatasen een belangrijke rol kunnen spelen in de moleculaire mechanismes betrokken bij de behandeling van kanker.

In Hoofdstuk 6 bestudeerden we een andere natuurlijke stof, ferruginol, die wordt gevonden in bomen van de familie Podocarpaceae. We toonden een kankerremmende werking aan van deze stof in de prostaat kanker cel lijn PC3. Analyse van de moleculaire mechanismen die hiervoor verantwoordelijk zijn liet zien dat ferruginol de activatie van PKB en ERK remt, en een blokkade in de G0/G1 fase van de celcyclus induceert. Behandeling van PC3 cellen met ferruginol leidt uiteindelijk tot apoptosis via 2 verschillende routes; intrinsiek (een verhoging van de ratio pro-apoptotische versus anti-apoptische eiwitten, nucleaire aanwezigheid van het eiwit AIF en activatie van caspase 9) en extrinsiek (verhoging van TNF-receptor expressie en caspase 8 activatie). De oxidatie status van de cel werd door ferruginol verhoogd, wat zijn weerslag vond op de phosphatase LMWPTP; zowel de expressie als de activiteit van deze werd door ferruginol negatief beïnvloed.

In zijn totaliteit laten de studies in dit proefschrift zien dat verschillende natuurlijke stoffen, voorkomend in groente, fruit, thee en bomen een kankerremmende werking kunnen hebben op verschillende types tumoren. Al naar gelang het type kanker en de natuurlijke stof die getest werden verschillende verantwoordelijke mechanismen hiervoor aangemerkt. In zijn algemeenheid kan gezegd worden dat remming van de activiteit van signaal transductie routes die belangrijk zijn in de overleving van cellen (PKB, ERK) worden een van de belangrijkste werkwijzen is van deze stoffen. De exacte manier waarop deze stoffen hun effect uitoefenen en de mogelijke rol die deze stoffen kunnen spelen in

de bestrijding van tumoren behoeven uiteraard nog veel onderzoek. Desalniettemin is het een aantrekkelijk idee om voor de behandeling van kanker terug te grijpen op de natuur, en gebruik te maken van stoffen die vrij voorhanden zijn, en voor een deel al in ons voedselaanbod voorkomen.

4. ACKNOWLEDGEMENTS

We are the sum of our experiences, social relationships and environment pressure. I reached this point of my life because of extraordinary people who gave me support, opportunities and happiness. Now is time to thank all of them.

First of all, I would like to thank my parents Lucelia and Pedro, who made all effort to give me a good education. For this reason, I studied in a good university that gave me a chance to study abroad. Here I am in the Netherlands, all my achievements I owe you both, who brought me up, taught me moral and ethical values and made me the person who I am. Thank you for your unconditional love, support, encouragement and care. Even so far away, I feel you both closer to me than ever. I love you!

My deep respect and thankfulness for my Brazilian mentors Prof. Dr. Hiroshi Aoyama and Prof. Dr. Carmen V. Ferreira. Prof Hiroshi, thank you for teaching me the basic steps of science, supporting me in my decisions and trusting me to lead our first grant together. Dear Carmen, I am very thankful for indicating me, as a representant of your lab, to work in P. Swaan's Lab in EUA and to M. Peppelenbosch's lab in Groningen, where I could learn a lot about research, science and different cultures. You are a fantastic scientist, an enthusiastic person, who gives to all your students strengh to go on during difficult times in science (bad results, disapointments etc.). Thank you both for reminding me my passion for science and support me in every moment of my professional carrier.

I would like to thank Prof. Dr. Maikel P. Peppelenbosch, my promoter in Groningen, for the opportunity to work in his lab. Maikel, I admire your brilliant mind, unbelievable ideas and fantastic solutions.

A special thank to Dr. Gwenny M. Fuhler, my co-promoter, teacher and friend. You were able to organize my ideas and to give me direction when I felt lost in my results. I am grateful to you for all your advices, practical solutions and discussions. Thank you very much for helping me in the writings, especially with the Dutch summary.

I would like to thank the reading committee, Prof. Dr. Bob Lowenberg, Prof. Dr. Paul Coffer, Prof. Dr. Nico Bos for reading and providing critical comments on my thesis.

Very big thank goes to Gerry and Greetje. Dear Gerry, thank you for helping me with all bureaucratic problems with visa, letters, contracts! You are a very kind person and very efficient professional! Greetje, if it was not for your efficiency, prompt response to all my desperate emails and enormous help with all

the paper work required for various procedures, I wouldn't be able to conclude this step.

To my work mates in the Cell Biology Department, section of Immunology for Ammerins, Annie, Davina, Eelke, Frans, Jeroen, Jacobus, Jan-Luuk, Karla, Kaushal, Lisa, Lu, Mirjam, Monireh, Nico, Sander, Rajesh, Sarah and Silvia for your help with equipament, search for reagents and nice talks during cake time!

I would like to thank all my collaborators Alba R. M. S. Brito, Ana C.S. Souza A. C., Arnold Spek, Carlos Areche, Eneida de Paula, Hiroshi Aoyama, Hela L. Aberson, Jaime A. Rodriguez, Jeroen Den Hertog, Karla C. S. Queiroz, Marcelo B. Jesus, Nel Blom, Guillermo Schmeda-Hirschmann and Willian F. Zambuzzi.

To my current workmates at AMC, for teaching me new subjects and techniques, having nice time together and providing nice work enviroment. Arnold, Keren, Lois, JanWillem, Hela, Karla, Kun and Lin, roommates from F0-117 and L01-145, thank you all!

Many thanks to all Brazilian colleagues who came to Groningen to work for few months and brought to me a bit of our Brazilian cheerfulness – Carmen, Giselle, William, Hugo, Alexandre, Luisa.

To my friends who made me feel at home in The Netherlands, who shared funny parties, unforgetable travels and spetacular dinners. Meeting you guys were one of the best thing that happenend to me in here! Thank you for make my life even better: Ana and Pedro, Kaushal and Maria, Mateusz and Marysia, Susana and Carlos, Karla and Arjan, Rajesh and Kavita, Ana, Guga, Gilda, Raquel, Adriana, thank you! All Brazilian girls, especially Grace, Cláudia, Daiana e Luciana, who I met in the monthly Portuguese-speaker meetings, thanks for your help and tips about The Netherlands. To my new Amsterdamer Brazilian family, Simone, Juca and the kids, Larissa, Heloísa and Elisa, thanks for accepting me your house, in your family, for the nice Brazilian barbecues and Brazilian birthday parties.

I would like to thank the very nice people from the 10th floor of Department of Cell Biology for nice parties and good time together: Ryuichi, Peter and Erika, Zia, Nai-Hua, Mira, Magda, Katica, Hande, Chris.

To all my friends back home, Anamaria, Bruna, Cacá, Gelvana, Julia, Lívia, Luli, Mariane, Michelle, Tati - thank you for all your continuous friendship beyond the distance.

To conclude, a special thank to Bispo, my love, my fiancé, my life. Thanks for your love, respect, patience and support. Thank you for giving me strength in difficult situation, cheering me up in depressing moments, celebrating my achivements, sharing amazing time in Brazil, in the Netherlands and all over places we have been together.

5. ABOUT THE AUTHOR

Roberta Regina Ruela de Sousa was born on June 1, 1982 in Campinas, SP, Brazil. She was raised in the same city and graduated of Liceu Salesiano Nossa Senhora Auxiliadora in 1999. During the last two years of high school she also attended Biochemistry Course at the Technical School ETECAP in Campinas, SP. In 1999 she did an internship in the Laboratory of Food Biochemistry under supervion of Dr. Helia Harumi Sato, in the College of Food Sciences, in State University of Campinas (UNICAMP). In 2000 she started the College of Biology at State University UNESP in Rio Claro, SP, Brazil. Her initiation in science started in 2002 in the Laboratory of Enzimology, at Department of Biochemisty, UNICAMP, under the supervision of Dr. Hiroshi Aoyama and financial support by FAPESP. She got a Master's Degree in Biochemisty, in 2005, whose dissertation was intitled "*Oxide-reduction studies of a protein tyrosine phosphatase (CD45) purified from human lymphocytes membranes*" with Scholarship provided by CAPES. She started her PhD at the same laboratory in 2006, with supervision of Dr. Hiroshi Aoyama and co-supervision of Dr. Carmen V. Ferreira. Together with Dr. H Aoyama, she got a 2-year grant for the project "*Role of Low Molecular Wight Protein Tyrosine Phosphatase in the progression of prostate cancer*". Two years later, she ended up in Groningen, the Netherlands, at the Department of Cell Biology section of Immunology, UMCG, due to a fruitful collaboration between Dr. Carmen V. Ferreira and Prof. dr. Maikel P. Peppelenbosch. The goal was to study signaling pathways induced by natural compounds in cancer cells. It was at UMCG where she finished her PhD in Medical Sciences under supervision of Prof. Dr. Maikel Peppelenbosch and co-supervision of Dr. Gwenny M. Fuhler.

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